La proteína transportadora de retinol tipo 4 y el retinol en la resección hepática con isquemia y reperfusión

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LA PROTEÍNA TRANSPORTADORA DE RETINOL TIPO 4 Y EL RETINOL EN LA RESECCIÓN HEPÁTICA CON ISQUEMIA Y REPERFUSIÓN

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Memoria presentada por Maria Elias Miró para optar al título de doctor por la Universidad de Barcelona.

La presente Tesis doctoral ha sido realizada bajo la dirección de la Dra. Carmen Peralta Uroz.

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Maria Elias Miró

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2013

"A hundred times every day I remind myself that my inner and outer life depends on the labors of other men, living and dead, and that I must exert myself in order to give in the measure as I have received and am still receiving."

Albert Einstein

A Pere Miró i Plans

A la meva familia i a en Jordi

PREFACIO

La presente tesis se enmarca dentro del estudio de la lesión por isquemia-reperfusión (I/R), y sus efectos sobre la regeneración, en hígados esteatósicos y no esteatósicos.
Uno de los principales riesgos en la cirugía hepática es la hemorragia que se produce durante las resecciones hepáticas. Para reducir este riesgo se realiza una oclusión vascular del flujo sanguíneo que entra en el hígado (isquemia). Si bien esto reduce el sangrado durante la cirugía, hay que tener presente las lesiones provocadas por la interrupción del flujo sanguíneo. Esta lesión hepática por isquemia, se agrava al restablecer el flujo sanguíneo (reperfusión). La esteatosis es la acumulación de cantidades excesivas de triglicéridos y otras grasas en las células hepáticas. Esta acumulación de grasa hepática es un factor de riesgo importante en la cirugía hepática, y se asocia con un mayor índice de complicaciones y mayor mortalidad postoperatoria. Se estima que más del 20% de pacientes programados para resección hepática presentan algún grado de esteatosis y, por lo tanto, es evidente la necesidad de desarrollar estrategias para minimizar los efectos adversos de la lesión por I/R en los hígados esteatósicos. En los últimos años, las investigaciones se han centrado en las adipocitoquinas como potenciales dianas terapéuticas en distintas patologías relacionadas con la obesidad y el síndrome metabólico; y en distintos antioxidantes para disminuir los daños por I/R hepática.

En la presente tesis se investigan el papel del RBP4 - adipocitoquina principalmente sintetizada en el hígado - y del retinol - potente antioxidante - en la lesión hepática por I/R y en el fallo en la regeneración asociada a la hepatectomía parcial en hígados esteatósicos y no esteatósicos.

La memoria consta de 6 capítulos, la bibliografía y un anexo. En la introducción se recopilan los antecedentes sobre resección hepática, lesión por I/R, las estrategias que se han establecido hasta el momento para reducir esta lesión y aumentar la regeneración, y las características de los hígados esteatósicos. El papel del RBP4 y del retinol, así como sus mecanismos de acción se discutirán también en esta sección. En el capítulo 2 se describen los objetivos de la presente tesis. En el capítulo 3 se detalla el diseño experimental y los materiales y métodos utilizados. En el capítulo 4 se describen los

resultados obtenidos; y en el capítulo 5 se discuten tales resultados. El último capítulo consiste en las conclusiones obtenidas a partir de los resultados de la presente tesis. La memoria finaliza con una lista exhaustiva de referencias que nos han permitido establecer las hipótesis de partida y complementan la información aportada; y un anexo donde se recogen los artículos principales que han dado lugar a esta tesis doctoral y la lista de artículos complementarios obtenidos en relación con los experimentos desarrollados en estos últimos años.

Agradecimientos: Ante todo quiero agradecer a mi directora de tesis, la Dra. C. Peraltra, por todo lo que he aprendido durante estos años que he trabajado bajo su dirección; también agradezco a mi tutora, la Dra. L. Baldomà, y a la coordinadora del master de doctorado, la Dra. J. Badia, por la ayuda prestada. Agradezco a mis compañeras de laboratorio Marta, Araní, Mariana, Laia y especialmente a Mónica, por el trabajo que hemos realizado en equipo y por los buenos momentos que hemos pasado juntas en el laboratorio y fuera de este. Agradezco al grupo del Dr. Parés, en especial a Silvia y a Marta, por las ayuda que me han brindado y las risas en el laboratorio. Agradezco a Ignasio, Loli y al resto del equipo del estabulario; y a Raquel y a Laura del BioBanco, por su colaboración técnica durante este tiempo. Agradezco a todos los grupos de la tercera planta del CEK, especialmente; al grupo del Dr. Bladimiro, sobre todo a Vedrana; al grupo del Dr. Fondevila; al grupo del Dr. Sánchez-Fueyo; y al grupo del Dr. Llovet; junto con la lab-manager de la planta, la Dra. J. Ros, por todas las ayudas prestadas.

También quiero agradecer al IDIBAPS y a la Fundación Clínic por las facilidades que me han dado y la agradable atmósfera de trabajo que han creado. También agradezco a todos a los que no he citado y me he podido olvidar.

Finalmente, agradezco a mis amigos, a toda mi familia y a Jordi por el soporte que siempre me han dado.

INDICE

ABREVIATURAS

ACE: Enzima conversora de la angiotensina

AP-1: proteína activadore 1

ATP: Trifosfato de adenosine

CE: células endoteliales

CINC: quimioatrayente de neutrófilos inducido por citoquinas 1

CitC: Citocromo C

CK: células de Kupffer

cNOS: NOS constitutiva

ENA-78: proteína activadora de neutrófilos derivada del epitelio 78

EGF: factor de crecimiento epidérmico

ET: endotelinas

GM-CSF: factor estimulante de colonias de granulocitos y macrófagos

GSH: glutatión

GSHPx: glutation peroxidasa

12-HETE: ácido 12-hidroxieicosatratraenoico

HIF-1: factor inducible por hipoxia-1

HMGB1: factor nuclear proteína de alta movilidad del grupo B1

H_2O_2: peróxido de hidrógeno

HO·: radical hidroxilo

HO-1: hemo oxigenasa-1

HSF: factor de transcripción de choque térmico

I/R: isquemia-reperfusión

ICAM-1: molécula de adhesión celular intercelular 1

INF-β: interferón beta

iNOS: NOS inducible

IFN-γ: interferón gamma

IGF-1: factor de crecimiento similar a la insulina 1

IL-1ra: antagonista del receptor de la IL-1

IL-1: Interleuquina 1

IL-6: Interleuquina 6

IL-8: Interleuquina 8

IL-10: Interleuquina 10

IL-12: Interleuquina 12

IL-13: Interleuquina 13

IL-18: Interleuquina 18

JNK: quinasa c-Jun N-terminal

KC: Quimioquina derivada de queratinocitos

LTB4: Leucotrieno B4

LRAT: lecitin:retinol acetiltransferasa

MAPK: Proteínas quinasas activadas por mitógenos

MCP: proteína quimiotáctica de monocitos

MCD: deficiente en metionina y colina

MIP: proteína inflamatoria de macrófagos

NAFLD: enfermedad del hígado graso no alcohólico

NASH: esteatohepatítis no alcohólica

NFκB: factor nuclear kappa B

NO: óxido nítrico

NOS: NO-sintasas

O_2^{-}: anión superóxido

$OONO^{-}\cdot$: peroxinitrito

p38 MAPK: Proteína quinasa activada por mitógeno p38 proteína quinasa activada por

PC: precondicionamiento isquémico

PAF: factor activador de plaquetas

PPAR-α: receptor activador de la proliferación de peroxisomas alfa

PPAR-γ: receptor activador de la proliferación de peroxisomas gamma

PUFA: ácidos grasos n-3 poliinsaturados

RBP4: Proteína transportadora de retinol tipo 4

RLO: radicales libres de oxígenos

SAPK: proteínas quinasa activadas

STAT3: transductor de señal y activador de la transcripción 3

STAT6: transductor de señal y activador de la transcripción 6

SLPI: inhibidor de proteasas secretado por leucocitos

SOD: superóxido dismutasa

TNF-α: factor de necrosis tumoral alfa

TNF-β: factor de necrosis tumoral beta

UCP-2: proteína desacoplante-2 mitocondrial

UW: Universidad de Wisconsin

VCAM-1: molécula de adhesión celular vascular 1

1.- INTRODUCCIÓN

1.1.- El hígado

1.1.1.- Generalidades

El hígado es un órgano glandular, accesorio del aparato gastrointestinal, al que se adjudica múltiples funciones metabólicas de vital importancia (**Tabla 1**). En adulto, representa del 2 al 5% del peso corporal y se localiza en el lado superior derecho de la cavidad abdominal, por debajo del diafragma. Además del papel del hígado en la detoxificación de productos de desecho del metabolismo, también actúa regulando el metabolismo energético al procesar los nutrientes que provienen de la digestión a fin de distribuirlos al resto de los tejidos. Otras funciones importantes del hígado son: la síntesis y secreción de bilis; la síntesis de proteínas, lipoproteínas plasmáticas y factores de coagulación; la detoxificación de fármacos y toxinas; y además, funciones metabólicas como son la síntesis de glucógeno, la gluconeogénesis y también el almacenamiento de glucógeno y de algunas vitaminas y lípidos (Fawcett D.W., 1997; Boyer T.D., y cols. 2003; Sherlok S., y cols. 2002).

Funciones principales del hígado
Metabolismo de carbohidratos: Captación de glucosa Síntesis y almacenamiento de glucógeno Gluconeogénesis y glucogenólisis
Metabolismo lipídico: Oxidación de ácidos grasos Síntesis de lipoproteínas, colesterol, triglicéridos y fosfolípidos Cetogénesis
Metabolismo proteico: Degradación de aminoácidos Síntesis de proteínas, ácidos nucleicos y urea
Metabolismo del grupo hemo: Síntesis del grupo hemo y de porfibrinógenos
Formación y secreción de bilis
Inactivación de diversas substancias como tóxicos esteroides y otras hormonas
Síntesis de proteínas plasmáticas: Albúmina y proteínas de fase aguda
Inmunidad: A través de las células de Kupffer

Tabla 1. Funciones principales del hígado

A nivel microscópico el hígado está organizado anatómicamente en unidades funcionales llamadas lóbulos. Los cuatro lóbulos, de distintos tamaños, están separados mediante una serie de tabiques de tejido conjuntivo (**Figura 1**).

- El lóbulo derecho, situado a la derecha del ligamento falciforme.
- El lóbulo izquierdo, situado a la izquierda del ligamento falciforme y extendido sobre el estómago.
- El lóbulo cuadrado, visible solamente en la cara inferior del hígado; se encuentra limitado por el surco umbilical a la izquierda, el lecho vesicular a la derecha y el hilio del hígado por detrás.
- El lóbulo de Spiegel o lóbulo caudato, situado entre el borde posterior del hilio hepático por delante y la vena cava por detrás.

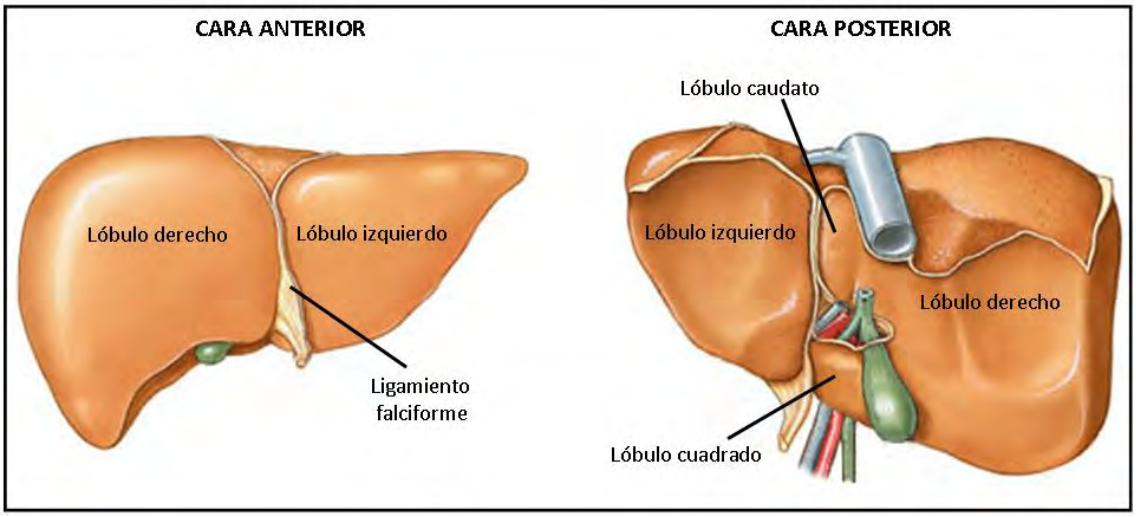


Figura 1. Esquema de los lóbulos hepáticos

Los lóbulos están formados por una estructura más o menos hexagonal de grupos de células epiteliales y hepatocitos, organizada alrededor de una vena central que desemboca en la vena hepática y a partir de la cual irradian columnas de hepatocitos distribuidos en forma de cordones. En los ángulos de estas zonas poligonales hay una pequeña área triangular formada por tejido conjuntivo que contiene un conducto biliar, una rama de la arteria hepática y una rama de la vena porta. Este complejo se denomina triada portal. Las ramas laterales de estos vasos confluyen en los sinusoides hepáticos, que ocupan los espacios que quedan entre los cordones de hepatocitos y drenan en la vena central. (**Figura 2**). Por lo tanto, las células hepáticas están expuestas a un gran volumen de sangre que fluye por los sinusoides. Por otro lado, la bilis es secretada

continuamente hacia una red de canalículos biliares situados en el interior de los cordones de hepatocitos y fluye hacia los conductos biliares situados en las triadas portales lípidos (Fawcett D.W., 1997; Byer T.D., y cols. 2003).

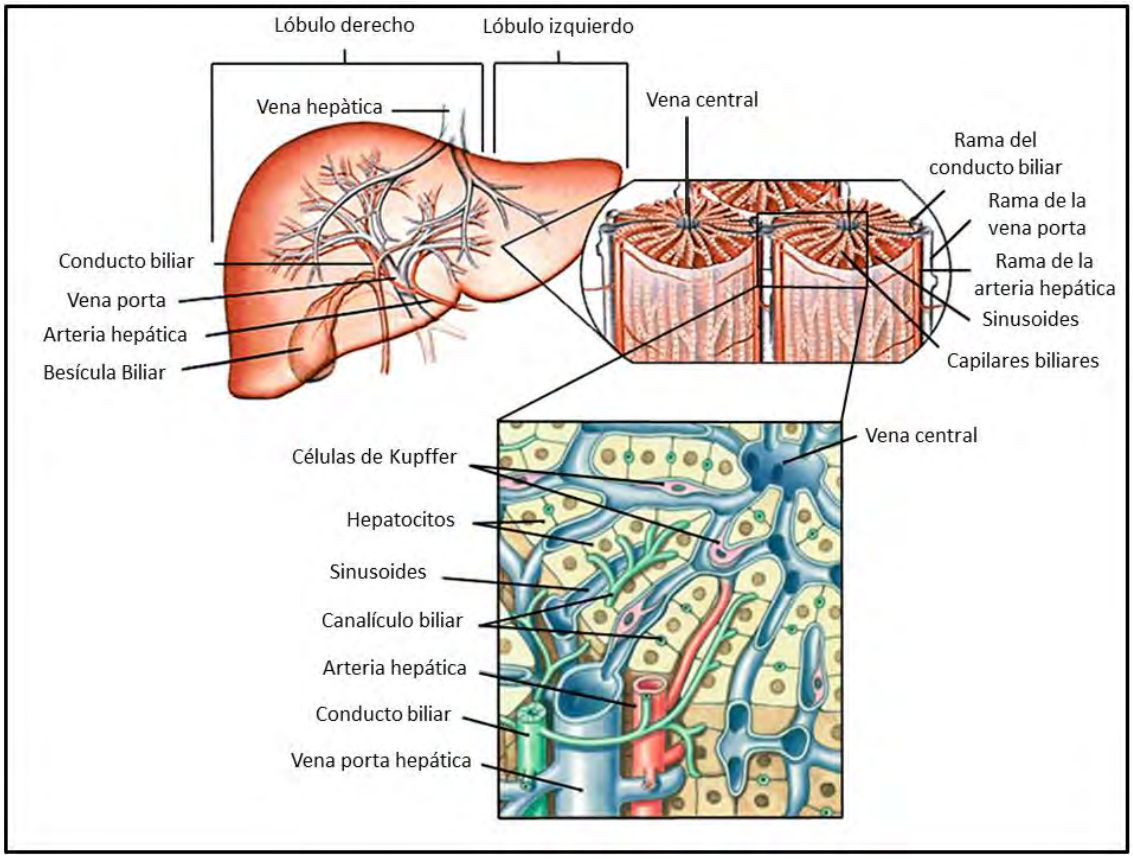


Figura 2. Estructura histológica del hígado

Se considera que el acino hepático es la unidad estructural y funcional básica del hepatocito (**Figura 3**). El acino hepático consiste en un área romboidal de hepatocitos cuyo centro es una tríada portal y los extremos son dos venas centrales (Fawcett D.W., 1997; Byer T.D., y cols. 2003; Sherlok S., y cols. 2002). A medida que la sangre arterial se acerca más a las venas centrales disminuye su calidad nutritiva, por lo que se distinguen tres zonas en cada acino:

- Zona 1, linda con el centro del acino y es la primera en recibir oxígeno.
- Zona 2, se encuentra en el medio.
- Zona 3, al lado de la vena centrolobular, donde hay mayor proporción de CO_2 y sustancias de desecho. Es la región más expuesta a las toxinas (Fawcet D.W., 1997).

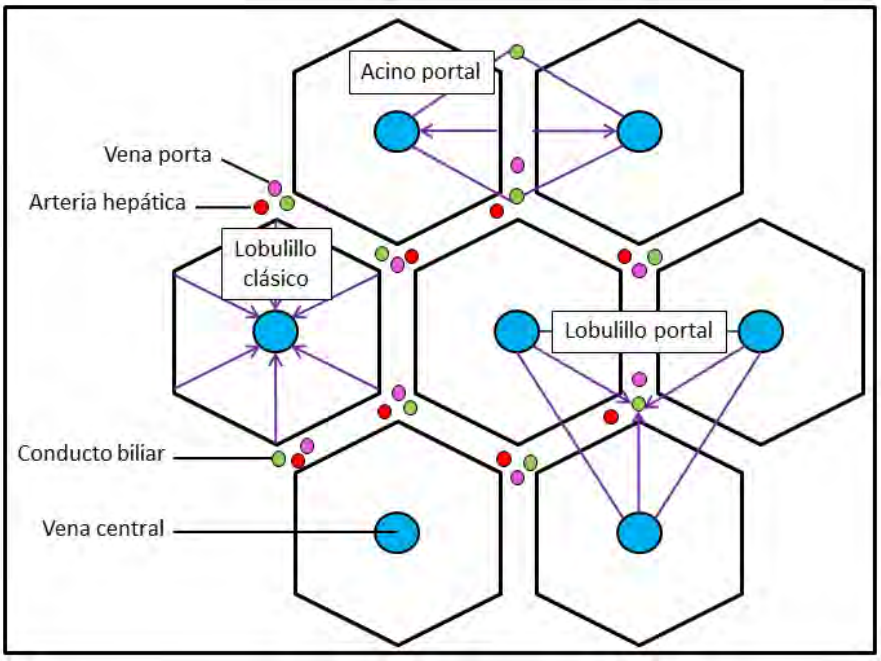


Figura 3. Estructura del lobulillo hepático

A diferencia de cualquier otro órgano, el hígado recibe sangre de dos vías diferentes (**Figura 4**):

- La vena porta (aproximadamente el 75% del aporte sanguíneo del hígado), que transporta la sangre proveniente del intestino, páncreas y bazo con un bajo contenido en oxígeno y rica en substancias absorbidas por el intestino, hormonas y otros elementos que se producen en el intestino, bazo y en el páncreas.
- La arteria hepática, que transporta la sangre rica en oxígeno procedente del corazón.

Tanto la sangre proveniente de la vena porta como de la arteria hepática alcanzará los sinusoides hepáticos, donde tiene lugar el intercambio entre la sangre y los hepatocitos. Los sinusoides hepáticos tienen una anchura superior a la de los capilares sanguíneos, y aunque su pared está formada por células endoteliales, hay zonas que permiten el acceso directo del plasma sanguíneo a los hepatocitos. Cada célula de los cordones hepáticos dispuestos radialmente está en contacto, al menos por un costado, con la sangre que fluye de los sinusoides hacia la vena central del lobulillo hepático, que desemboca en las vénulas hepáticas y finalmente en la vena cava inferior justo por debajo del diafragma (Fawcett D.W., 1997; Boyer T.D., y cols. 2003; Sherlok S., y cols. 2002). De esta manera, tal y como se ha comentado anteriormente, las características de la sangre

que llega a cada hepatocito es diferente según su distancia de la vena portal, y trae como consecuencia una gran heterogeneidad funcional entre los hepatocitos.

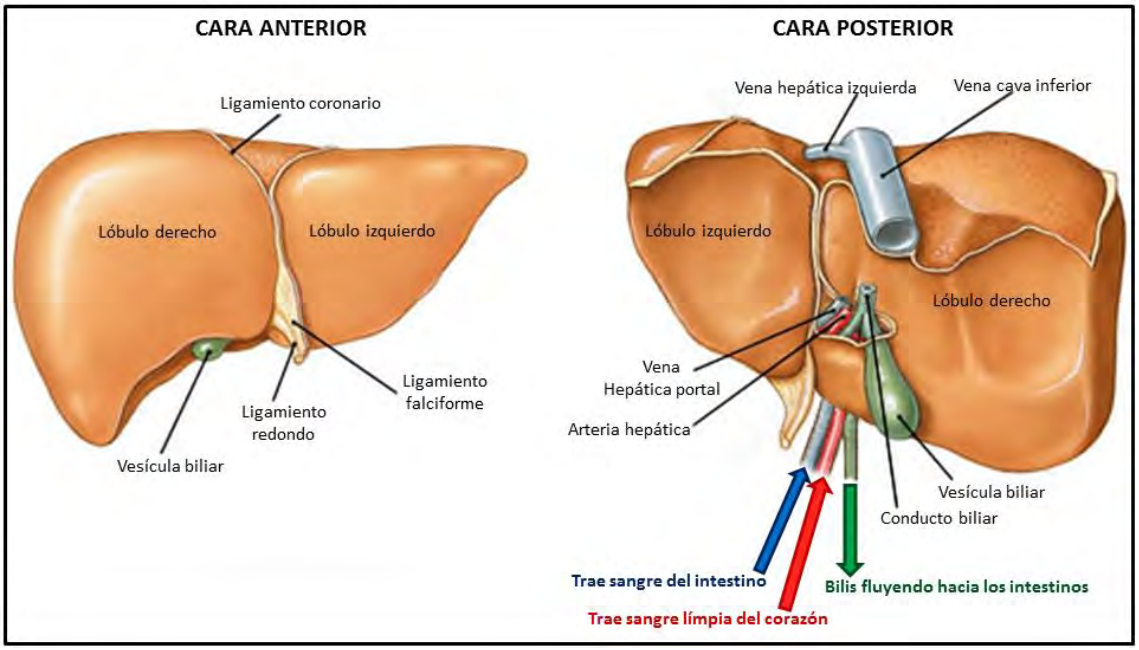


Figura 4. Irrigación sanguínea del hígado

El hígado se puede dividir siguiendo un criterio funcional; así la vena porta se bifurca en dos ramas que dividen el hígado en dos partes, la izquierda y la derecha. Cada mitad tiene un aporte sanguíneo independiente, tanto desde la vena porta como desde la arteria hepática; de la misma manera, el drenaje biliar también es independiente. Cada una de las dos ramas de la vena porta se bifurca en dos, dividiendo el hígado en cuatro sectores, que a su vez se dividen en 8 segmentos (**Figura 5**). Cada segmento tiene su propio pedículo vascular y biliar, así como su drenaje venoso. La clasificación de este modo facilita la realización de resecciones hepáticas y las técnicas de trasplante parcial (Cingolani H.E., y cols. 2000; Ganong W.F., 2001; Pocock G., y cols. 2002; Sherlock S., y cols. 2002; Boyer T.D., y cols. 2003).

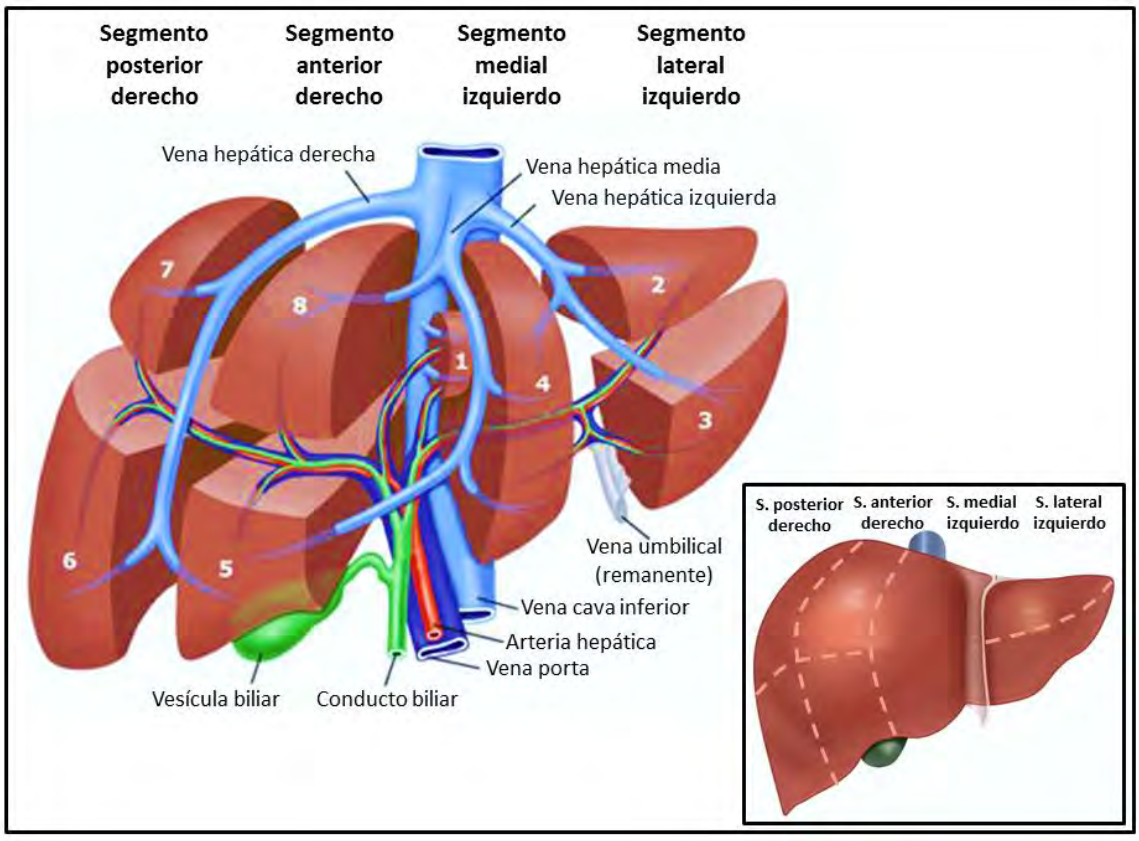


Figura 5. Esquema de la segmentación hepática del hígado en su cara anterior o diafragmática.

1.1.2.- Células hepáticas

1.1.2.1.- Hepatocitos

Los hepatocitos son las células del parénquima hepático y representan aproximadamente el 90% de la masa hepática total. A nivel metabólico son una de las células más complejas y diversas del organismo, y llevan a cabo una gran variedad de funciones, entre las que se incluyen: la obtención, almacenamiento y liberación a la circulación sanguínea de un gran número de nutrientes (glúcidos, proteínas, lípidos y vitaminas); la síntesis de proteínas plasmáticas, lipoproteínas, glucosa, ácidos grasos, colesterol y fosfolípidos; la digestión y absorción de las grasas alimenticias a través de la secreción de la bilis; y la detoxificación de compuestos endógenos y exógenos tóxicos para el organismo (Boyer T.D., y cols. 2003) (**Figura 6**).

A pesar de que tienen una forma poliédrica, se pueden distinguir dos dominios funcionalmente importantes:

- La superficie apical o canalicular está especializada en el transporte de sales biliares, bilirrubina, colesterol, fosfolípidos, así como xenobióticos en la bilis.
- El dominio basolateral que está en contacto con la sangre y rodea el espacio de Disse. Este dominio de la membrana del hepatocito está formado por numerosas microvellosidades que permiten un aumento sustancial de la superficie de intercambio. Además, su membrana plasmática contiene muchas proteínas de transporte transmembrana.

A causa de la alta tasa metabólica, los hepatocitos tienen un gran número de orgánulos citplasmáticos. Entre ellos destaca:

- Retículos endoplasmático, donde se sintetizan proteínas, se realiza el metabolismo lipídico, el metabolismo de la glucosa y la degradación del grupo hemo.
- Aparato de Golgi, donde tiene lugar la síntesis de proteínas, la glicosilación de proteínas, el reciclaje de receptores y la secreción de bilis.
- Mitocondrias, donde tiene lugar la fosforilación oxidativa y la síntesis de trifosfato de adenosina (ATP) (Sherlok S., y cols. 2002).

1.1.2.2.- Células endoteliales

Las células endoteliales forman el revestimiento endotelial de los sinusoides hepáticos y están separadas de los hepatocitos por el espacio de Disse. Estas células poseen características únicas y actúan como barreras entre la corriente de la sangre por un lado y los hepatocitos en el otro. Se distinguen de otras células endoteliales ya que entre ellas existen poros o fenestraciones de gran tamaño, permitiendo el intercambio de fluidos y compuestos macromoleculares entre los sinusoides y el espacio de Disse (Fawcett D.W., 1997; Boyer T.D., y cols. 2003; Sherlock S., 2002). Las células endoteliales participan activamente en la inflamación, de manera que frente a determinados estímulos pueden liberar diversos mediadores como interleuquinas y óxido nítrico (NO) (Boyer T.D., y cols. 2003; Peralta C., y cols. 2013) (**Figura 6**).

Las células endoteliales también juegan un papel importante en parte de los procesos del hígado. Son conocidas por su secreción de citoquínas, tales como el TNF-α y la IL-6 (Nagano T., y cols. 1992; Knolle P.A., y cols. 1997); y expresan la molécula de

adhesión celular intercelular 1 (ICAM-1) y molécula de adhesión celular vascular 1 (VCAM-1), que son cruciales en la interacción con los leucocitos y los neutrófilos.

1.1.2.3.- Células de Kupffer

Las células de Kupffer son los macrófagos del hígado y se encuentran localizadas principalmente en la pared de los sinusoides hepáticos, emitiendo prolongaciones que se extienden hacia la luz de los sinusoides y entre las células endoteliales subyacentes. Además de su ubicación estratégica, las células de Kupffer se caracterizan por su alta actividad fagocítica, actuando como barrera protectora eliminando diversas sustancias potencialemte dañinas para el organismo tales como microorganismos, endotoxinas, complejos inmunes, eritrocitos envejecidos o lesionados, células tumorales, lípidos, etc. La activación de las células de Kupffer puede estar provocada por diferentes estímulos, entre ellos las endotoxinas. Una vez activadas, generan mediadores citotóxicos como son citoquinas, radicales libres de oxígeno (RLO) y proteasas (Boyer T.D., y cols. 2003; Peralta C., y cols. 2013) (**Figura 6**).

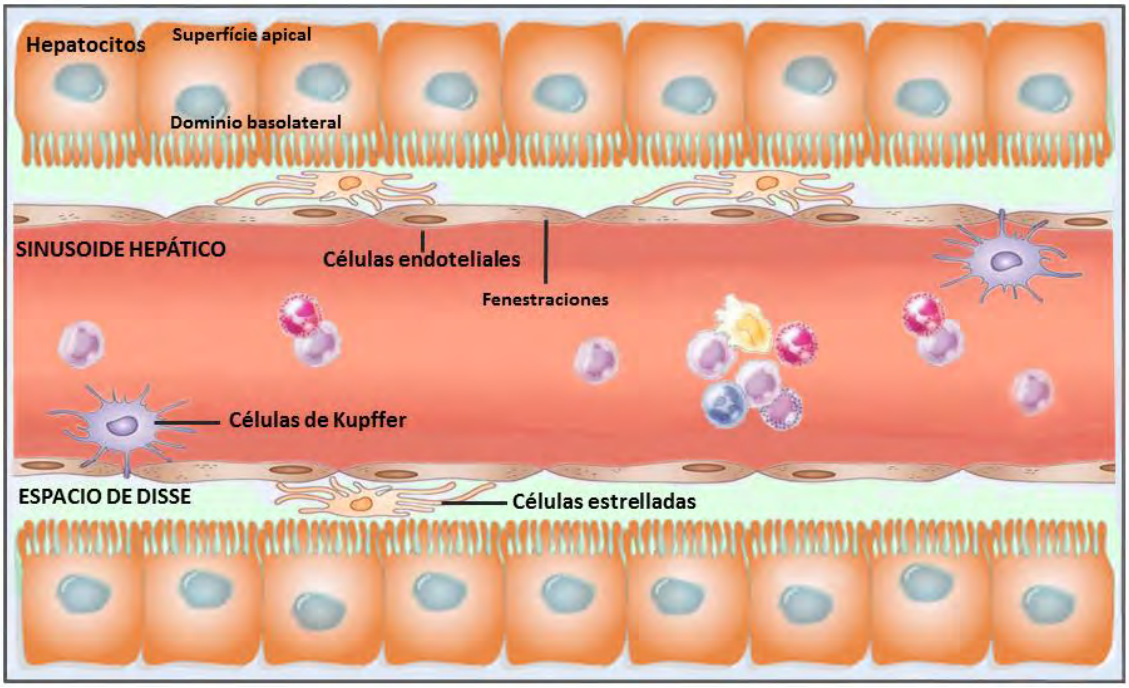


Figura 6. Esquema de la disposición de las células hepáticas.

1.1.2.4.- Células estrelladas

Las células estrelladas, también conocidas como células de Ito, están localizadas en el espacio de Disse, en estrecho contacto con las células endoteliales, y constituyen el principal almacén de vitamina A del organismo. Uno de sus rasgos característicos es la

expresión de dos fenotipos diferentes. Como consecuencia de esta capacidad de transformación, las células estrelladas también cambian sus funciones. Las células estrelladas sintetizan proteínas de la matriz extracelular (colágenos de distintos tipos), son mediadores importantes de procesos de reparación del tejido hepático en diversas patologías hepáticas y poseen capacidad para promover y amplificar la respuesta inflamatoria (Boyer T.D., y cols. 2003; Peralta C., y cols. 2013).

1.2.- La resección hepática

Una resección hepática es la extirpación quirúrgica de la totalidad o una parte del hígado. También se conoce como hepatectomía, total o parcial.

La mayoría de hepatectomías parciales se realizan para el tratamiento de neoplasias hepáticas, tanto benignas o malignas. Las neoplasias benignas incluyen adenoma hepatocelular, hemangioma hepático e hiperplasia. Los tumores nodulares focales malignos más comunes (cáncer) del hígado son las metástasis; las derivadas de cáncer colorrectal se encuentran entre las más comunes, y más susceptible de resección quirúrgica. El tumor maligno primario más común del hígado es el carcinoma hepatocelular. La hepatectomía también puede ser el procedimiento de elección para el tratamiento de cálculos biliares intrahepáticos o quistes parasitarios del hígado.

Las resecciones hepáticas completas se realizan en el contexto de un trasplante, donde se extrae un hígado enfermo y se implanta otro procedente de donante cadavérico. En la mayoría de los trasplantes procedentes de donante cadavérico se trasplanta todo el hígado (hepatectomía total). En cambio, los trasplantes de donante vivo solo se puede implantar un segmento de tejido hepático (injerto de tamaño reducido), que se adquiere a través de una hepatectomía parcial.

1.3.- La regeneración hepática

Una de las características principales del hígado es su capacidad de regenerarse tras una agresión o resección. Esta capacidad regenerativa no deja de sorprender ya que el hígado es un órgano quiescente, en términos de proliferación celular (Koniaris L.G., y cols. 2003), en el cual solamente menos del 0,01% de los hepatocitos se están dividiendo en un momento determinado. Esta regeneración hepática se da mediante una proliferación masiva de los hepatocitos supervivientes tras una lesión o resección hepática, y cesa cuando la masa del hígado llega a representar una fracción determinada del peso del individuo, que varía según la edad y la especie (Koniaris L.G., y cols. 2003). Por ejemplo, si a un paciente se le extrae el 40% del hígado, este recupera la masa perdida a los seis meses. Aunque los hepatocitos son los primeros en proliferar, todos los tipos celulares hepáticos se dividen, desde las células epiteliales biliares, las células endoteliales, hasta las células de Kupffer (Michalopoulo G.K., y cols. 1997). Inmediatamente después del estímulo regenerativo, los hepatocitos salen del estado G_0 para entrar en la fase G_1. Luego entran en la fase S, dónde dividen su DNA y entran en mitosis (fase M). En las ratas el pico de regeneración se da a las 24 horas, y existe un segundo pico menor entre las 36 y las 48 horas (Koniaris L.G., y cols. 2003; Michalopoulo G.K., y cols. 1997). Los demás tipos celulares entran en división 24 horas después que los hepatocitos.

La regeneración hepática se ha dividido en dos epatas:

1) La salida del hepatocito de la fase quiescente para entrar en el ciclo celular (iniciación).
2) La progresión a través del punto de restricción de la fase G_1 del ciclo (progresión).

Se ha propuesto que estas dos etapas tienen un control independiente. En la primera etapa, la iniciación, intervendrían las citoquinas (TNF-α y IL-6). En la progresión intervendrían los factores de crecimiento [el factor de crecimiento hepatocitario (HGF) y el factor de crecimiento transformante β (TGF-β)] (Fausto N. 2000) (**Figura 7**).
En la regeneración intervienen diferentes citoquinas y factores de crecimiento de los cuales nos centraremos en los de mayor relevancia, como son la IL-6, el TNF-α, el HGF, el TNF-β y la IL-1. Las células no parenquimales del hígado tienen una función

importante como fuente de factores de crecimiento e interleuquinas que promueven e inhiben la proliferación de los hepatocitos; aunque existen controversias sobre el papel individual de cada población.

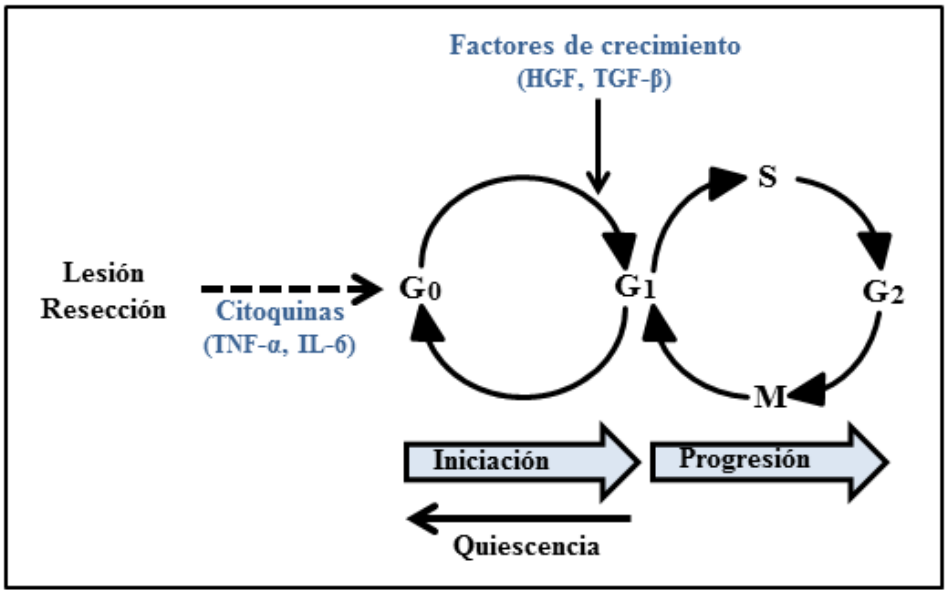


Figura 7. Modelo de regeneración hepática en diferentes etapas

Las células no parenquimales que forman parte de la arquitectura del hígado tienen un papel muy importante en la liberación de todas estas moléculas. Las células de Kupffer son responsables tanto de la producción y liberación de las citoquinas proliferantes TNF-α e IL-6, como de las anti-proliferantes IL-1 y TGF-β (Malik R., y cols. 2002; Taub R. 2004). Las células endoteliales de los sinusoides, aunque no son la fuente mayoritaria de citoquinas, son capaces de generar TGF-β, HGF e IL-6. Por otro lado, las células estrelladas hepáticas sintetizan y liberan HGF y TGF-β (Malik R., y cols. 2002; Taub R. 2004).

1.3.1.- HGF

El HGF, que fue aislado gracias a su habilidad de inducir la síntesis de DNA en cultivos de hepatocitos, actúa mediante el receptor c-met tirosina quinasa (Michalopoulo G.K., y cols. 1997). Diferentes estudios *in vivo* han demostrado sus efectos inductor de la regeneración hepática y su aumento plasmático cuando se produce una disminución de la masa hepática (Yamada Y., y cols. 1997; Michalopoulo G.K., y cols. 1997). En un estudio basado en un modelo experimental de trasplante hepático en rata, la administración de HGF dio lugar a un aumento de la regeneración hepática del injerto

(Uchiyama H., y cols. 1999); mientras que en otro estudio, con un modelo experimental de regeneración por tetracloruro de carbono, la administración de anticuerpos anti-HGF inhibió la regeneración hepática (Burr A.W., y cols. 1998).

1.3.2.- TGF-β

Como ya se ha comentado anteriormente, el TGF-β es un conocido inhibidor de la proliferación de hepatocítica. El TGF-β pertenece a una superfamilia con más de 30 miembros. Este factor de crecimiento es sintetizado como una pre-pro-molécula que requiere varias modificaciones para su activación. Esta pre-pro-molécula está formada por dos dímeros:

a) TGF-β madura; dímero que procede del extremo C-terminal.
b) LAP; dímero que procede del extremo N-terminal, que está asociado a la latencia del TGF-β.

Estos dos dímeros interactúan para facilitar el tránsito del TGF-β y asegurando la inactividad de este. El LAP se desprende en condiciones de pH extremos, calor y proteasas, entre otros (Khalil N., 1999) (**Figura 8**).

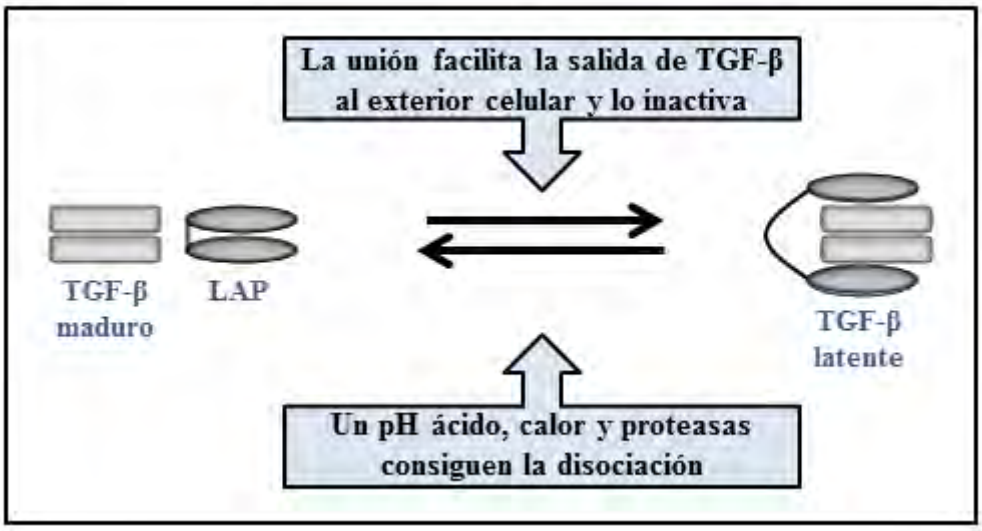


Figura 8. Interacción entre el TGF-β y el péptido asociado a la latencia (LAP).

La síntesis del TGF-β empieza a las pocas horas de una hepatectomía, llegando a su máxima concentración alrededor de las 48 horas. La administración intravenosa de TGF-β inhibe la fase temprana de la regeneración hepática después de una hepatectomía en rata (Russell W., y cols. 1988), mientras que el tratamiento con anticuerpos anti-

TGF-β de ratas con una hepatectomía parcial da luar a un aumento en la regeneración hepática (Enami Y., y cols. 2001).

1.3.3.- Células de Kupffer

Durante la isquemia fría las células de Kupffer sufren alteraciones, que conllevan a su activación cuando se inicia la reperfusión. Esta activación de las células de Kupffer va acompañada de una liberación de gran cantidad de mediadores inflamatorios, como RLO, TNF-α, proteasas, IL-1, IL-6, entre otros (Lemasters J.J., y cols. 1983). Distintos estudios han reportado la implicacón de las células de Kupffer en la lesión por I/R. Se ha observado que la administración de galdgadolinium ($GdCl_3$), que es un potente inhibidor de la activación de las células de Kupffer, atenúa la lesión hepática por I/R. Por otro lado, la administración de partículas de látex, activadoras de la actividad de las células de Kupffer, agrava la lesión asociada a este proceso (Klein A.S., y cols. 1996). De todas formas, existen controversias sobre el papel de las células de Kupffer en la regeneración hepática tras una resección, ya que se ha demostrado que una depleción de las células de Kupffer antes de una resección hepática promueve o inhibe la regeneración del hígado dependiendo del estudio y del modo de inhibir dichas células (Malik R., y cols. 2002).

1.4.- La lesión por isquemia-reperfusión hepática

1.4.1.- Introducción

La lesión por isquemia-reperfusión (I/R) afecta negativamente la regeneración hepática. Es un proceso complejo que se inicia cuando el hígado se ve privado temporalmente de flujo sanguíneo (isquemia) y se agrava al restablecerse el flujo sanguíneo (reperfusión). La lesión por I/R fue reconocida como un desorden patológico clínicamente relevante por Toledo-Pereyra y cols. en 1975 realizando estudios en trasplante hepático experimental (Teoh N.C., y cols. 2003).

En la práctica clínica tiene lugar la isquemia normotérmica y la isquemia fría, dependiendo de la temperatura a la cual está sometido el hígado durante la isquemia hepática. Así pues, se habla de isquemia normotérmica cuando el hígado se mantiene a 37ºC. La lesión por I/R normotérmica es clínicamente relevante en cirugía hepática, trasplante hepático, shock hipovolémico, algunos tipos de daño tóxico hepático, enfermedad veno-oclusiva y síndrome de Budd-Chiari. Por otro lado, la isquemia fría hace referencia a la situación en la cual el hígado es sometido a una temperatura de 4ºC cuando se ve privado de aporte sanguíneo. La lesión por I/R fría ocurre durante la preservación del hígado antes del trasplante y se aplica intencionalmente para reducir la actividad metabólica del injerto mientras él se mantiene en la solución de preservación hasta el implante. Los mecanismos y tipos celulares implicados en la lesión por I/R son diferentes dependiendo de si la isquemia es fría o caliente. En los años 80 se demostró que la I/R fría específicamente causaba daño a las células endoteliales sinusoidales. La lesión en las células endoteliales favorece la adhesión leucocitaria y de plaquetas, lo cual induce fallos en la microcirculación hepática. En numerosos modelos experimentales se ha demostrado que el grado de lesión en las células endoteliales se correlaciona con la duración de la isquemia fría. A diferencia de lo que ocurre en condiciones de isquemia fría, la I/R normotérmica afecta a todos los tipos celulares hepáticos, tales como hepatocitos, células endoteliales, macrófagos, leucocitos adherentes y plaquetas (Selzner M., y cols. 2003).

1.4.2.- Relevancia quirúrgica de la I/R

La hemorragia durante la cirugía del hígado es el riesgo principal durante las resecciones hepáticas (Foster J.H., y cols. 1977; Nagao T., y cols. 1987; Nagorney

D.M., y cols. 1989; Makuuchi M., y cols.1989; Delva E., y cols. 1989). Para reducir este riesgo se utiliza la oclusión vascular del flujo sanguíneo que entra en el hígado, esta maniobra se conoce como la maniobra de Pringle u oclusión de la tríada portal. Consiste en la compresión del ligamento hepatoduodenal (Pringle J.H., 1908). La maniobra de Pringle permite minimizar el sangrado de la superficie cortada durante la resección del parénquima, aunque no tiene efecto sobre el sangrado de las ramas de las venas hepáticas (Huguet C., y cols. 1992; Selzner M., y cols. 2000). Otra técnica que se utiliza, es la exclusión vascular hepática, consiste en la oclusión de la tríada portal y la de la vena cava inferior. Esta técnica aísla completamente el hígado y la vena cava retrohepática del resto de la circulación, y tiene como objetivo reducir el riesgo de hemorragia masiva y de formación de trombos gaseosos causados por la ruptura de la vena cava, o de la vena hepática, al extraer un tumor hepático muy extenso o bien situado en el lóbulo posterior del hígado (Huguet C., y cols. 1992). Con el fin de minimizar el sangrado durante la cirugía hepática y las alteraciones bioquímicas ocasionadas por la interrupción del flujo sanguíneo del hígado, Makuuchi y sus colaboradores desarrollaron una técnica basada en el control selectivo de los vasos del híleo hepático, llamada oclusión vascular hemihepática (Makuuchi M., y cols. 1987; Miyagawa S., y cols. 1996; Yanaga K., y cols. 1993; Nishizaki T., y cols. 1996). Esta técnica consiste en la oclusión de los vasos aferentes (arteria hepática y vena porta) de los lóbulos hepáticos, derechos o izquierdos dependiendo de la situación del fragmento hepático a reseccionar, de manera que se preserva el flujo sanguíneo en el lóbulo hepático contralateral reduciendo así la lesión hepática asociada a la I/R (Miyagawa S., y cols. 1996; Yanaga K., y cols. 1993; Nishizaki T., y cols. 1996). Si bien estos procedimientos reducen el sangrado durante la cirugía, hay que tener presente las lesiones provocadas por la interrupción del flujo sanguíneo en la totalidad del hígado. Además, en pacientes con cirrosis hepática los dos tipos de oclusiones vasculares comportan congestión intestinal, con el consiguiente riesgo añadido de sepsis (Makuuchi M., y cols. 1987).

En otro contexto quirúrgico, como es el trasplante hepático procedente de donante vivo, tiene lugar una I/R junto con una resección hepática parcial. La técnica del trasplante de donante vivo fue utilizada para receptores pediátricos y en 1994 se realizó el primer trasplante hepático procedente del lóbulo derecho de un donante vivo. Actualmente esta modalidad quirúrgica representa una alternativa válida y segura en centros con experiencia en cirugía hepática llegando a representar el 25% del total de los trasplantes

realizados (Marcos A., y cols. 1999). Uno de los beneficios de los injertos de tamaño reducido a partir de donante vivo es obtener un injerto de buena calidad con un tiempo isquémico corto, ya que la cirugía del donante puede ser programada para coincidir en tiempo con la cirugía del receptor. Por otro lado, el mayor problema concerniente a la aplicación del trasplante hepático de donante vivo es la disparidad del tamaño del injerto, lo cual implica una posterior regeneración del injerto hepático de tamaño reducido hasta alcanzar el tamaño estándar. Es conocido, que esta posterior regeneración se ve empeorada en hígados esteatósicos, pudiendo dar problemas postquirúrgicos (Otte J.B., y cols. 1998; Tanaka K., y cols. 1993; Ozawa K., y cols. 1992; García-Valdecasas J.C., y cols. 2003).

1.4.3.- Patofisiología de la lesión hepática por I/R

En la literatura, la lesión por I/R se ha dividido en dos fases, la lesión causada por la isquemia y la causada por la reperfusión. Esta separación de los eventos celulares no es absoluta, ya que el daño celular en el órgano hipóxico se acentúa después de la restauración del aporte de oxígeno, lo que sugiere que los eventos que suceden en la reperfusión, son la consecuencia de aquellos que se inician durante la isquemia. Además se ha visto que la reperfusión de un hígado expuesto a un periodo de isquemia breve no induce ningún daño detectable, un hallazgo indicativo de que la reperfusión por sí sola no es perjudicial (Casillas-Ramírez A., y cols. 2006a).

Durante la isquemia, se interrumpe el aporte de oxígeno al hígado y se detiene la cadena respiratoria mitocondrial, lo que comporta una depleción de los niveles ATP. La degradación de ATP estimula la glucólisis anaeróbica con la consiguiente formación de ácido láctico. La acidosis resultante además de alterar la cinética normal de las enzimas, resulta ser un sistema menos efectivo para producir ATP y las células se ven privadas de la energía necesaria para mantener la homeostasis. El fallo en la homeostasis celular se caracteriza por la pérdida de gradiente de los iones de sodio y de calcio a través de las membranas celulares. Este hecho trae como consecuencia edema intracelular y el consiguiente hinchamiento de las células de Kupffer y las células endoteliales. Estos fenómenos inducen una alteración en los orgánulos citoplasmáticos y en la integridad de la membrana, pudiendo desencadenar en la muerte celular (Casillas-Ramírez A., y cols. 2006a).

La reperfusión (recuperación del flujo sanguíneo) del hígado previamente isquémico inicia toda una serie de fenómenos inflamatorios en los que están implicados múltiples

mediadores de la inflamación, plaquetas, leucocitos y el endotelio vascular, los cuales al interaccionar derivan en la lesión por reperfusión. A título de ejemplo, entre los mediadores inflamatorios descritos en la lesión por I/R hepática destacan los RLO, interferón beta y gamma (INF-β y IFN-γ), interleuquinas 1, 12 y 18 (IL-1, IL-12 e IL-18), factor de necrosis tumoral alfa y beta (TNF-α y TNF-β), factor estimulante de colonias de granulocitos y macrófagos (GM-CSF), leucotrieno B4 (LTB4), ácido 12-hidroxieicosatratraenoico (12-HETE), y el factor activador de plaquetas (PAF). Además, también se ha demostrado la participación de interleuquinas con propiedades anti-inflamatorias que funcionan como reguladores del proceso inflamatorio que se desarrolla en la I/R, estas son las interleuquinas 6, 10 y 13 (IL-6, IL-10 e IL-13). Los mediadores inflamatorios son modulados a nivel transcripcional. Distintos estudios sobre transducción de señales en I/R hepática han descrito un papel notorio para diferentes factores de transcripción tales como el factor nuclear kappa B (NFκB), proteína activadora 1 (AP-1), receptor activador de la proliferación de peroxisomas alfa (PPARα), factor nuclear proteína de alta movilidad del grupo B1 (HMGB1), STAT3 y STAT6, factor inducible por hipoxia-1 (HIF-1), y factor de transcripción de choque térmico (HSF). En la lesión por reperfusión también están implicadas las quinasas intracelulares que activan factores de transcripción, como proteínas quinasa activadas (SAPK), quinasa c-Jun N-terminal (JNK) o proteína quinasa activada por mitógeno p38 (p38 MAPK) (Casillas-Ramírez A., y cols. 2006a).

La acumulación de neutrófilos, asociada a la lesión por I/R está regulada por la participación tanto de citoquinas, factores del complemento, moléculas de adhesión y quimioquinas que permiten el reclutamiento, adhesión y transmigración de neutrófilos. Las moléculas de adhesión celular conocidas por su papel en I/R hepática son E, selectina, P-selectina, L-selectina, integrinas-β1, integrinas β2, ICAM-1 y VCAM-1. De la misma manera, se ha sugerido la participación de quimiocinas, entre las que destacan la interleuquina 8 (IL-8) y sus homólogos, el quimioatrayente de neutrófilos inducido por citoquinas 1 (CINC), la proteína activadora de neutrófilos derivada del epitelio 78 (ENA-78), la proteína inflamatoria de macrófagos (MIP) 1 y 2, la quimiocina derivada de queratinocitos (KC) y la proteína quimiotáctica de monocitos (MCP) 1, 2 y 3.

Por último, la disfunción microvascular asociada a la lesión por I/R hepática resulta de una serie de eventos que implican la interacción de células intravasculares (por ejemplo neutrófilos) con células no parenquimales, (como las células endoteliales y las células de Kupffer) y que es mediada por la síntesis y liberación de moléculas de adhesión,

citoquinas, factores de complementos, RLO, NO y endotelinas (ET) (Casillas-Ramírez A., y cols 2006a).

Lo anteriormente expuesto evidencia la multitud de mediadores y factores implicados en la lesión por I/R hepática (**Figura 9**). Las interrelaciones entre estos medidores y sus respectivas vías de señalización son muy complejas y aún no es posible hablar con total certeza de los eventos que suceden desde que se inicia la reperfusión hasta el momento en que se evidencian los fallos postoperatorios asociados a la cirugía hepática, pues las diversas investigaciones que abordan el tema no han logrado converger en sus resultados. Este hecho se ve acentuado negativamente por la gran diversidad de modelos y diseños experimentales con los que se trabaja.

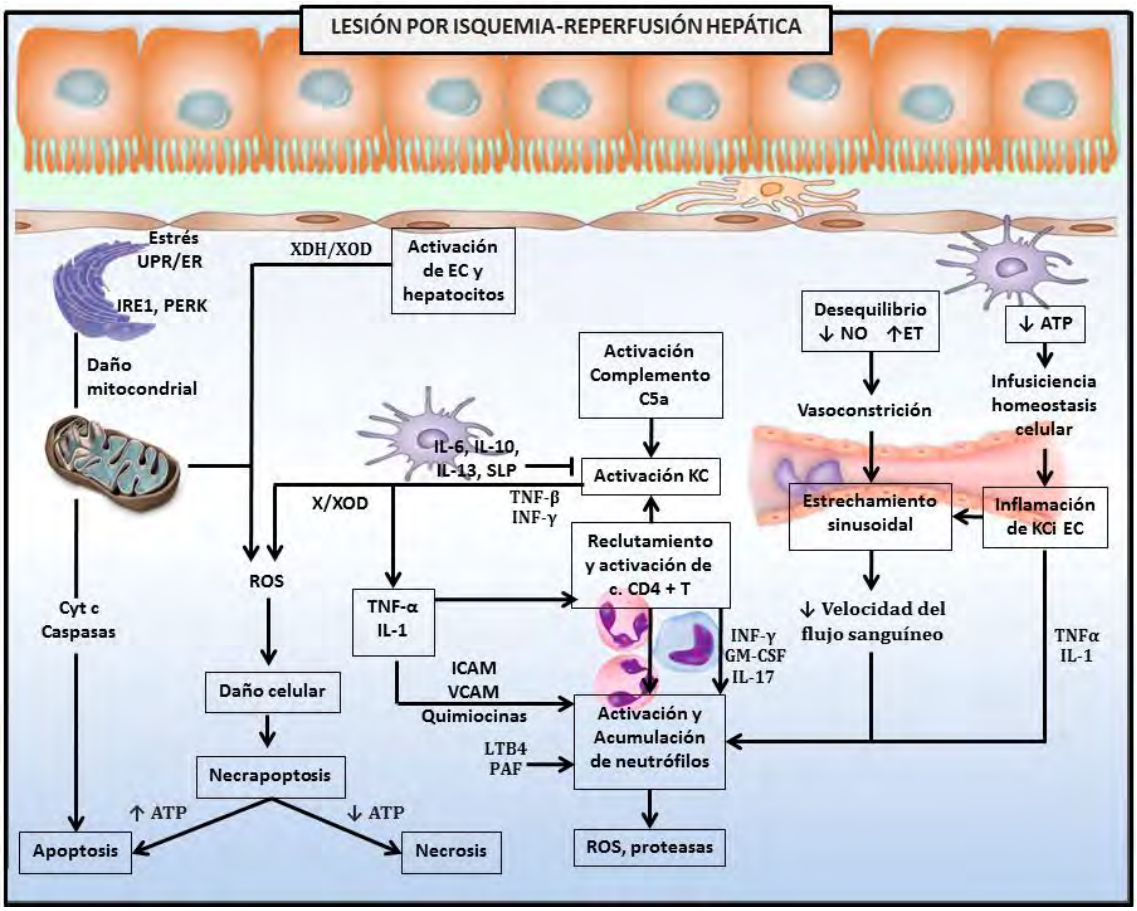


Figura 9. Mecanismos implicados en la patofisiología de la lesión por I/R hepática. La deficiencia energética inducida por la isquemia resulta en el fallo del transporte activo transmembranal y consecuentemente se producen cambios en la morfología de las células de Kupffer (CK) y de las células endoteliales (CE). Este hecho junto con el desequilibrio en la producción de NO y ET contribuye al estrechamiento del lumen sinusoidal. Esto conduce tanto a una acumulación de neutrófilos como a una disminución en la perfusión. La activación de las CK resulta en la liberación de RLO, TNFα e IL-1. Adicionalmente, los RLO pueden producirse a partir del sistema xantina /xantina oxidase X/XOD y de la mitocondria. La liberación de citoquinas a través de la inducción de ICAM y VCAM, y de la quimioquina CXC conduce a la acumulación y activación de neutrófilos. Estos neutrófilos se extravasan causando daño parequimal a través de la producción de RLO y proteasas. De la misma manera, la activación del factor del complemento C5a prepara y activa a las CK; los linfocitos T CD4+ residentes y los recién acumulados en el hígado pueden producir GM-CSF, INF y TNF-β, los cuales amplifican la activación de las CK y promueven el reclutamiento de neutrófilos en el hígado; el PAF puede preparar a los neutrófios para la generación de superóxido mientras que el LTB4 puede contribuir a la amplificación de la respuesta de los neutrófilos. Adicionalmente, factores anti-inflamatorios tales como IL-6, IL-10, IL-13 y el inhibidor de proteasas secretado por leucocitos (SLPI) atenúan el daño por reperfusión. Finalmente, la teoría de la necroapoptosis postula que un proceso puede empezar con una señal común de muerte celular y culminar en muerte celular necrótica o en apoptosis, dependiendo del grado de disminución del ATP celular.

1.4.4.- Factores implicados en la lesión por I/R y regeneración

A continuación nos centraremos en los diferentes resultados existentes en la literatura acerca de posibles fuentes generadoras de RLO, de los efectos y mecanismos de acción del NO, del papel de determinados mediadores pro-inflamatorios como el TNF y de la activación y reclutamiento de neutrófilos. Estos datos nos permitirán entender aún más por qué la I/R hepática continúa siendo un problema sin resolver en la práctica clínica.

1.4.4.1.- Óxido Nítrico (NO)

El NO es una molécula gaseosa producida por la familia de las NO-sintasas (NOS) que convierten la L-Arginina, en presencia de oxígeno, en L-citrulina. En la lesión por I/R es difícil establecer una distinción entre mediadores que tienen un papel beneficioso de aquellos cuyo papel es perjudicial. Algunos autores han observado que el NO ejerce un efecto beneficioso frente al daño por I/R en diferentes órganos, tejidos y células, mientras que otros estudios no destacan efecto alguno del NO, e incluso reportan una acción perjudicial de este mediador vasoactivo en el daño por I/R hepática. Estos efectos diferenciales del NO se pueden explicar por las diferencias en las enzimas productoras de NO (Casillas-Ramírez A., y cols. 2006b).

En este contexto, algunos estudios sugieren que aunque la producción de NO derivada de la NOS constitutiva (cNOS) tendría efectos beneficiosos en I/R hepática, la producción de NO derivada de la NOS inducible (iNOS), contribuiría al daño hepático. El NO derivado de la isoforma cNOS, constitutivamente expresada, puede disminuir los desórdenes de la microcirculación durante las primeras horas de reperfusión. En cambio, el NO derivado de la iNOS no puede ser generado hasta varias horas después de la reperfusión debido a que esta isoforma requiere inducción transcripcional. El exceso de producción de NO derivado de la iNOS ya no sería beneficioso a la microcirculación a ese tiempo de reperfusión, ya que se pueden combinar con los RLO y generar peroxinitrito. Además, hay estudios que proponen que el NO derivado de la iNOS induce la liberación de Citocromo C (CitC) y de caspasas favoreciendo los procesos pro-apoptóticos. Sin embargo, también existen controversias en relación al papel del NO derivado de la iNOS, ya que otros investigadores han sugerido que este NO es beneficioso porque induce la expresión de la proteína anti-apoptótica Bcl-2 e inactiva caspasas (Casillas-Ramírez A., y cols. 2006b).

Además, para entender los diferentes resultados en relación al papel del NO en la lesión por I/R hepática, es importante clarificar si la fuente de NO es endógena o exógena. Mientras que se ha demostrado que el NO endógeno puede reducir la acumulación de neutrófilos, la suplementación exógena de NO no modificó este parámetro pero se asoció con una inhibición en la producción de ET (Casillas-Ramírez A., y cols. 2006b).
El desequilibrio entre el NO y la producción del anión superóxido representan uno de los mecanismos responsables del estrés oxidativo en el daño por I/R. En condiciones fisiológicas normales, tanto el NO como el anión superóxido son producidos en las células endoteliales, con una producción de NO superior a la del anión superóxido en 2/3 órdenes de magnitud. Esto permite al NO:

1) Captar eficazmente anión superóxido intracelular,
2) Prevenir la agregación plaquetaria,
3) Minimizar las interacciones entre los neutrófilos y las células endoteliales.

Tras la I/R hepática, los niveles de anión superóxido exceden a los del NO debido a una sobreproducción de anión superóxido y/o a una biodisponibilidad de NO reducida drásticamente. Este desequilibrio puede ser debido a:

1) Niveles intracelulares bajos de cofactores necesarios para la síntesis de NO (NADPH y oxígeno),
2) Degradación de L-arginina por la gran cantidad de arginasa liberada tras la isquemia,
3) La inhibición de la actividad NO sintasa de las células endoteliales, y
4) La rápida inactivación del NO por el anión superóxido. Por lo tanto, en estas condiciones las acciones beneficiosas del NO no se evidencian (Elias-Miró M., y cols. 2012a).

En cuanto a la influencia del NO sobre la regeneración hepática se ha demostrado que el NO es necesario para la viabilidad de los hepatocitos (Fausto N. 2000) y que juega un papel importante en la repoblación hepatocitaria del hígado (Koniaris L.G., y cols.2003). La cNOS y la iNOS parecen jugar un papel importante en la regeneración del hígado después de una hepatectomía. Tras una hepatectomía, el hígado remanente recibe el volumen de flujo sanguíneo que recibía el hígado entero y esto lleva a una

mayor producción de NO por parte de la eNOS en las células endoteliales, lo cual es escencial para que las células del parénquima hepático entren en la fase G_1 y empiece la regeneración hepática. Por otro lado, parece ser que la iNOS tendría un papel importante 6-8 horas después de la hepatectomía, y su inducción dependería de citoquinas (TNF-α e IL-6) (Koniaris L.G., y cols.2003).

1.4.4.2.- Radicales libres de oxígeno (RLO)

Los radicales libres se definen como moléculas, átomos, o iones que contienen uno o más electrones desapareados. Entre los radicales libres de oxígeno destacan los radicales: anión superóxido (O_2^-), el peróxido de hidrógeno (H_2O_2), el radical hidroxilo ($HO^·$) y el peroxinitrito ($OONO^-$). El H_2O_2 no es estrictamente un RLO ya que no tiene un electrón libre, pero se considera junto con los RLO por su capacidad de formar radicales hidroxilo (Elias-Miró M., y cols. 2012a).

A causa de estos electrones desapareados los RLO son especies muy reactivas que pueden captar o ceder electrones muy fácilmente. La reacción de un radical libre con un no-radical puede generar un radical libre diferente que puede ser más o menos reactivo que el original. Como consecuencia, las reacciones producidas por los radicales libres provocan reacciones en cadena que pueden extender el daño considerablemente. De esta manera pueden producir lesión a través de la modificación de nucleótidos, carbohidratos o lípidos. Parece que sus efectos más nocivos se producen a través de su acción sobre las membranas celulares, ya sea a nivel de enzimas, receptores de membrana, mecanismos de transporte o modificando las propiedades de lípidos y proteínas de dichas membranas. También pueden desencadenar los procesos de peroxidación lipídica de los ácidos grasos poliinsaturados, afectando directamente las propiedades físicas de la membrana y provocando una depleción de estos ácidos grasos esenciales (Horrobin D.F., 1991; Ferguson D.M., y cols. 1991, Kanno M., y cols. 1993; Elias-Miró M., y cols. 2012a).

El estrés oxidativo que acompaña la lesión por I/R hepática es la consecuencia de un desequilibrio entre sustancias pro-oxidantes y antioxidantes. Entre los mecanismos que generan sustancias pro-oxidantes destacan la generación mitocondrial, la generación por parte de los fagocitos y la del sistema xantina deshidrogenasa/xantina oxidasa (XDH/XOD). En cuanto a los sistemas antioxidantes destacan la superóxido dismutasa (SOD) y el glutatión (GSH) como sistemas enzimáticos y no-enzimáticos respectivamente. En la lesión por I/R se ven afectados los sistemas antioxidantes que

podrían eliminar los RLO. En este sentido la I/R provoca una disminución de los niveles de SOD y GSH, hecho que agrava aún más los efectos nocivos de los RLO.

Los RLO, al dañar proteínas y DNA tienen una influencia negativa sobre la división celular (Clavien P.A., y cols. 1992; Diesen D.L., y col. 2010). Distintos estudios indican que los marcadores de peroxidación lipídica están aumentados durante la reperfusión en trasplante así como en resecciones hepáticas (Biasi F., y cols. 1995; Reilli P.M., y cols. 1991). La modulación farmacológica de los citados mecanismos pro-oxidantes (basados en la administración de un inhibidos de XOD, como el alopurinol o en el pretratamiento de antioxidantes, como la SOD) confirman sus efectos perjudiciales en la regeneración hepática (Portugal V., y cols. 1995).

Sistemas generadores de RLO:

- Generación por fagocitos: La producción extracelular de RLO por parte de los macrófagos y de los neutrófilos es un mecanismo fisiológico del que disponen los fagocitos para lisar células bacterianas. Pero en determinadas ocasiones como ocurre en I/R hepática, este mecanismo puede estar implicado en lesiones tisulares, tal como tiene lugar en la lesión por I/R. En este sentido, la isquemia activa las células de Kupffer que liberan RLO durante la primera fase de reperfusión, además, durante esta fase tiene lugar el reclutamiento de neutrófilos, los cuales, en la fase posterior, se activan generando más RLO vía NADPH oxidasa (Metzger J., y cols. 1988; Drath D.B., y cols. 1975).

- Sistema X/XOD: Durante la isquemia, debido a la degradación de ATP, se produce una acumulación de xantina e hipoxantina, que son los substratos de la XDH y de la XOD (Stirpe F., y cols. 1969; Atalla S.L., y cols. 1985; McCord J.M., 1985). Al mismo tiempo, la regulación de los niveles de calcio se ve afectada, el calcio aumenta en el citoplasma y activa a las proteasas citoplasmáticas dependientes de calcio que transforman la XDH en XOD (Atalla S.L., y cols. 1985; McCord J.M., 1985). En el momento de la reperfusión, tiene lugar un aporte considerable de oxígeno que conduce a la oxidación rápida de la xantina y la hipoxantina y a la formación del radical $O2^{-\cdot}$, el cual mediante determinadas reacciones dará lugar al $OH\cdot$ (Atalla S.L., y cols. 1985; McCord J.M., 1985). El $O2^{-\cdot}$ dada su elevada reactividad es capaz de inducir procesos de peroxidación lipídica, favoreciendo la permeabilidad de la membrana celular y liberando enzimas hidrolíticos que extenderán el daño tisular. El radical $O2^{-\cdot}$ actúa

además como agente quimiotáctico para los neutrófilos, que se activan y producen aún más radicales libres, amplificando la lesión en el órgano. (Clavien P.A., y cols. 1992).

- Generación mitocondrial: En determinadas sitiuaciones como es la I/R, la cadena respiratoria se puede ver afectada y entonces se desencadena una sobreproducción de RLO. En este sentido, durante el periodo de isquemia de un tejido, la anoxia asociada hace que la cadena de transporte electrónico mitocondrial se pare y se produzca una caída de los niveles energéticos celulares. Durante la reperfusión del tejido, se producen unas alteraciones en las mitocondrias que llevan a la formación de aniones superóxido, que conducen a la pérdida del potencial de membrana mitocondrial (Nieminen A.L., y cols. 1995; Nieminen A.L., y cols. 1997; Elias-Miró M., y cols. 2012a). Resultados obtenidos en modelos experimentales de hígado perfundido aislado han puesto en tela de juicio la importancia del sistema X/XOD, e indican que la mitocondria podría ser la fuente principal de RLO. Sin embargo, datos obtenidos en un modelo murino de I/R normotérmica *in vivo* constatan que la mitocondria no parece participar activamente en el estrés oxidativo inducido por la reperfusión (Casillas-Ramírez A., y cols. 2006b; Elias-Miró M., y cols. 2012b).

La divergencia de resultados respecto a los mecanismos de generación de RLO en la I/R hepática es evidente. Con el objeto de clarificar la importancia del sistema X/XOD frente a la mitocondria, debe tenerse en cuenta las diferencias existentes entre los modelos experimentales evaluados, incluyendo los tiempos de isquemia. De esta manera, el sistema X/XOD desempeña un papel crucial en el daño hepático por I/R solamente en condiciones en las cuales ocurre una formación importante de XOD, como por ejemplo a las 16 h de isquemia fría. Sin embargo, este sistema de generación de RLO no parece ser crucial en períodos isquémicos más cortos tales como 6 h de isquemia fría. (Casillas-Ramírez A., y cols. 2006b; Elias-Miró M., y cols. 2012a). Por otro lado, también deben considerarse los fármacos usados para inhibir el sistema X/XOD, ya que, el alopurinol por ejemplo, parece tener más de un mecanismo de acción. El alopurinol no es solamente un inhibidor potente de la XOD, sino que también mejora la disfunción mitocondrial inducida por la isquemia. De la misma manera, es importante señalar que la implicación de los hepatocitos y/o de las células de Kupffer como fuentes productoras de RLO depende del periodo de isquemia y de la temperatura (4ºC y 37ºC) a la cual se somete el hígado durante la isquemia, lo cual probablemente conduce a diferentes mecanismos de daño en la I/R hepática (Casillas-Ramírez A., y cols. 2006b; Elias-Miró M., y cols. 2012a).

Sistemas de defensa frente al estrés oxidativo:

- Mecanismos enzimáticos:

- Superóxido dismutasa: La superóxido dismutasa es el primer enzima de la vía de detoxificación de los RLO y se encuentra presente en el citoplasma y en el espacio intermembrana de las mitocondrias. La SOD cataliza la reacción entre dos O_2^- y da lugar a una molécula de H_2O_2, de esta forma disminuye la cantidad de radical O_2^-.

La síntesis de SOD está regulada a nivel genético y puede modificarse en respuesta a concentraciones de oxígeno altas, generación de radicales superóxido o disponibilidad de metales.

Durante un proceso de I/R hepática se produce el aumento de radicales O_2^-, provocando la inactivación de la SOD (Peralta C., y cols. 2002a). Este hecho acentúa aún más los efectos nocivos de los RLO. En modelos experimentales de trasplante hepático la sobreexpresión de la SOD protege frente al estrés oxidativo y al daño inducido por la I/R hepática (Lehmann T.G., y cols. 2003).

- Catalasa: Este enzima cataliza la descomposición del H_2O_2 a agua. La célula la actividad catalasa se encuentra confinada en orgánulos subcelulares, principalmente en los peroxisomas, pero también se puede encontrar catalasa no peroxisomal en células de ciertos tejidos (Link E.M., 1990; Mavier P., y cols. 1988).

- Peroxidasas: De todas las peroxidasas, la más importante es la glutation peroxidasa (GSHPx), enzima que se localiza en el citosol y la matriz mitocondrial de las células de la mayoría de las especies. Cataliza el paso de glutation reducido a glutation oxidado.

- Mecanismos no enzimáticos:

- Glutation: El glutation es un tripéptido formado por los aminoácidos: ácido glutámico-cisteína-glicina. Juega un papel importante en su protección frente al daño oxidativo actuando como secuestrador de RLO y como sustrato de la glutation peroxidasa (Koken T., y cols. 1999). Existe en forma reducida (GSH) y en forma oxidada (GSSG); en la forma oxidada dos moléculas de glutation se unen a través de un puente disulfuro formado entre los grupos tiol de la cisteína. El paso de GSH a GSSG consume peróxido de hidrógeno, neutralizando esta molécula. El cociente GSH/GSSG es indicativo del estrés oxidativo de un tejido, así cuando este cociente es alto, el tejido cuenta con defensas ante los RLO, y

cuando éste disminuye el tejido está sometido a un estrés oxidativo que hace que predomine la forma oxidada del glutation. Tras someter a un órgano a un proceso de I/R se ha observado que disminuyen los niveles de GSH. Este descenso de GSH aumenta la vulnerabilidad de la célula frente al estrés oxidativo inducido por la I/R hepática (Koken T., y cols. 1999; Peralta C., y cols. 2002a).

- Otros antioxidantes: Las células disponen de otras sustancias antioxidantes con capacidad de reaccionar directamente con los RLO. Entre estos antioxidantes se encuentran la vitamina C, la vitamina E o tocoferol, los β-carotenos, el ácido úrico y los flavonoides (Serracino-Inglott F., y cols. 2001).

1.4.4.3- Neutrófilos:

Al inicio de la reperfusión, la acumulación de neutrófilos en el hígado viene determinada por distintos cambios celulares que incluyen, entre otros, la producción de citoquinas como la IL-1 y el TNF-α por las células de Kupffer y el aumento de la expresión de las moléculas de adhesión como la ICAM-1 (Ghezzi P., y cols. 1991; Zhou W., y cols. 1992). Una vez los sinusoides se han activado liberan RLO, lesionando tanto las células endoteliales como los hepatocitos, induciendo así una disfunción microvascular y el daño parenquimal asociado a la I/R hepática (Serracino-Inglott F., y cols. 2001; Jaeschke H., y cols. 1992; Lentsch A.B., y cols. 2000).

Actualmente, se desconoce cómo se acumulan los neutrófilos en el hígado. La teoría clásica propone el aumento en la expresión de moléculas de adhesión, tales como ICAM-1 y P-selectina. Oponiéndose a esta teoría, también hay resultados que demuestran que la acumulación de neutrófilos observada en el hígado después de la I/R no depende de la sobreexpresión de ICAM-1 o P-selectina (Elias-Miró M., y cols. 2012a; Elias-Miró M., y cols. 2012b; Casillas-Ramírez A., y cols, 2006b; Luscinskas F.W., y cols.1991; Issekutz T.B., 1990).

Tales resultados estarían de acuerdo con la teoría propuesta por Jaeschke. Esta teoría propone que aunque la P-selectina y el ICAM-1 parecen ser relevantes en la adherencia de los neutrófilos en las vénulas postsinusoidales, los neutrófilos relevantes en el daño por I/R se acumulan en los sinusoides, que han sido identificados como los sitios dominantes para la extravasación de neutrófilos. En estos capilares, la acumulación de neutrófilos no depende de B2 integrinas, de ICAM-1 ni de selectinas. En estos sinusoides, los neutrófilos se acumulan gracias a factores mecánicos como la

vasocontricción, el daño y el edema de las células que forman la pared vascular, y una reducida flexibilidad de la membrana del neutrófilo. Tales factores contribuyen a la acumulación de neutrófilos sin la necesidad de un aumento en la expresión en las moléculas de adhesión como el ICAM-1 (Elias-Miró M., y cols. 2012b; Casillas-Ramírez A., y cols. 2006b).

La lesión vascular durante la reperfusión elimina, en parte, la barrera de células endoteliales sinusoidales y los neutrófilos tienen entonces acceso directo a los hepatocitos. Sin embargo, incluso con células endoteliales dañadas y aún presentes, puede haber transmigración. Como consecuencia, en estas condiciones, las terapias anti-ICAM pueden reducir pero no previenen la lesión por I/R. respecto al papel de la P-selectina, las células endoteliales no contienen cuerpos de Weibel Palade ni tampoco sobrerregulan transcripcionalmente niveles importantes de P-selectina.

Por otro lado, durante la I/R, un número de intervenciones dirigidas contra las selectinas reducen la acumulación hepática de neutrófilos y la lesión celular. Debido a que estos resultados, se ha sugerido que la mayoría de los modelos de I/R hepática incluyen algún grado de isquemia intestinal, la cual conduce a la acumulación de neutrófilos en órganos remotos, incluyendo el hígado. Por lo tanto, un bajo número de neutrófilos en el hígado tras bloquear las selectinas puede ser un efecto secundario debido a la protección que ejerce la terapia anti-selectina en el daño intestinal por reperfusión (Elias-Miró M., y cols. 2012b; Casillas-Ramírez A., y cols. 2006b).

Tal y como se ha comentado anteriormente, los neutrófilos activados lesionan tanto las células endoteliales como los hepatocitos mediante la liberación de proteasas y de RLO (Bilzer M., y col. 1994; Jaeschke H., y cols. 1992; Lentsch A.B., y cols. 2000; Serracino-Inglott F., y cols. 2001). La liberación de mediadores inflamatorios por parte de los neutrófilos agrava la lesión por I/H y afecta negativamente el proceso de regeneración hepática. Estos datos se basan en el hecho de que la administración de anticuerpos dirigidos a inactivar la acción de los neutrófilos disminuye el daño hepático en animales sometidos a I/R (Arndt H., y cols. 1991; Palma-Vargas J.M., y cols. 1997; Suzuki S., y col. 1993; Vollmar B., y cols. 1995), mientras que la infusión directa de neutrófilos en hígados aislados sometidos a I/R aumenta la formación de RLO y la lesión hepatocitaria (Weiss S.J., 1989).

1.4.3.4.- Citoquinas:

Las citoquinas son un grupo de proteínas producidas por diferentes células, que actúan como moléculas de señalización celular.

Las citoquinas pro-inflamatorias más importantes son la IL-1α, IL-1β y el TNF-α. Las principales acciones de estas tres citoquinas en la inflamación son los efectos sobre el endotelio, los leucocitos y los fibroblastos, así como la inducción de reacciones sistémicas de fase aguda. Tal y como se ha descrito anteriormente, en la I/R hepática provocan la activación del endotelio, induciendo la síntesis de IL-8, ICAM-1 y de selectinas, aumentando las interacciones entre los leucocitos y las células endoteliales Elias-Miró M., y cols. 2012a; Elias-Miró M., y col., 2012b; Casillas-Ramírez A., y cols. 2006b; Luscinskas F.W., y cols. 1991, Issekutz T.B., y cols. 1990).

La citoquina pro-inflamatoria TNF puede ser protectora o perjudicial dependiendo del tipo celular y de las condiciones experimentales, pudiendo estimular la muerte celular o inducir efectos hepatoprotectores. Por ejemplo, se ha constatado su implicación en la regeneración hepática en trasplantes hepáticos con injertos de tamaño reducido (Casillas-Ramírez A., y cols. 2006b).

La IL-10, citoquina principal anti-inflamatoria en la lesión por I/R, es mayoritariamente producida por linfocitos T, linfocitos B y macrófagos. Se sabe que esta citoquina anti-inflamatoria reduce la activación de macrófagos inhibiendo la síntesis de citoquinas pro-inflamatorias como la IL-1α, IL-1β y el TNF-α, e inhibiendo también la producción de RLO durante la primera fase de la inflamación (Moore K.W., y cols. 1993; D´Andrea A., y cols. 1993; Bussolatti B., y cols. 1997; Gerard C., y cols. 1993).

El TNF-α, que se ha demostrado que juega un papel en la regeneración hepática, actúa distintamente según la dosis en que administre. La administración de dosis altas de TNF-α resulta en una pobre regeneración hepática, mientras que no tiene efecto alguno si se administra en dosis bajas. Por otro lado, esta citoquina induce la síntesis de DNA en cultivos de hepatocitos en presencia de suero, pero sin efecto mitogénico alguno cuando los cultivos de hepatocitos son mantenidos en un medio libre de factores de crecimiento. Todas estas observaciones sugieren que el TNF-α actúa como un agente responsable de la iniciación de la proliferación hepatocitaria en la primera fase de regeneración hepática, dejando las células preparadas para responder a los factores de crecimiento (Fausto N. 2000; Yamada Y., y cols. 1997).

En el contexto de la regeneración hepática, se ha visto que la IL-6 está íntimamente ligada al TNF-α. Estudios con ratones knock-out para los genes TNFR1 y IL-6,

mostraron una pobre regeneración tras una hepatectomía parcial, y una posterior administración de IL-6 revertía los efectos de la inhibición de la vía de señalización del TNF-α. A partir de estos estudios se sugirió que el TNF-α, a través del TNFR1, podría iniciar la regeneración hepática y activar una vía dependiente de IL-6 en la que participa el factor de transcripción; transductor de señal y activador de la transcripción 3 (STAT3) (Yamada Y., y cols. 1997). Además de estas observaciones hay que resaltar que existen otros trabajos en hepatectomía parcial con knock-out para TNFR1, que demostraron que la proliferación hepática podía ser inducida por dos vías diferentes, una dependiente de TNF-α e IL-6, y una inducida por mitógenos, independiente de estas citoquinas (Ledda-Columbano G.M., y cols. 1998).

La IL-1 también está implicada en la regeneración hepática. Se ha demostrado que tanto la IL-1α, como la IL-1β tienen una acción inhibitoria sobre la proliferación de hepatocitos *in vitro* (Boulton R., y cols. 1997; Court F.G., y cols. 2002). En estudios *in vivo* basados en la hepatectomía en rata, se ha visto que la administración exógena de IL-1β produce una disminución de la regeneración hepática (Boulton R., y cols. 1997).

1.5.- El hígado esteatósico

1.5.1.- Definición y prevalencia

La esteatosis o hígado graso es la acumulación de cantidades excesivas de triglicéridos y otras grasas dentro de las células del hígado. Esta acumulación de grasas, es considerada patológica cuando excede el 5% del peso del hígado (Veteläinen R., y cols. 2007). La evaluación cuantitativa se basa en el porcentaje de hepatocitos que muestran vacuolas lipídicas en su citoplasma y se clasifica de la siguiente forma:

1) Leve, menos de un 30% de los hepatocitos contiene grasa;
2) Moderada, entre un 30% y un 60% de los hepatocitos contienen grasa; y
3) Severa, más de un 60% de los hepatocitos contienen grasa.

La evaluación cualitativa se basa en el tipo de esteatosis y puede dividirse en dos tipos:

1) Macrovesicular, cuando los hepatocitos contienen una única vacuola grande que desplaza el núcleo del hepatocito hacia la periferia; y
2) Microvesicular, si contienen múltiples vacuolas pequeñas.

Las circunstancias más habituales asociadas a esteatosis hepática es la enfermedad crónica del hígado. Varios estudios han reportado una prevalencia del 10%-20% en la población delgada, 60-74% entre la población obesa y más de 90% en obesos mórbidos. Aproximadamente un 3% de los niños delgados están afectados y la prevalencia incrementa hasta un 53% en niños obesos. Debido al aumento de la obesidad en los países occidentales, se espera que en el futuro aumente la prevalencia de esteatosis hepática (Veteläinen R., y cols. 2007).

1.5.2.- Importancia de la esteatosis hepática en la cirugía hepática

La esteatosis hepática es un hallazgo histológico común en biopsias de hígado humano, y se estima que más del 20% de los pacientes programados para resección hepática presentan algún grado de esteatosis (Veteläinen V., y cols. 2007). En posibles donantes de hígado, la prevalencia de esteatosis hepática es del 26% (Selzner M., y cols. 2001). La esteatósis hepática es un factor de riesgo importante en la cirugía hepática, y se asocia con un mayor índice de complicaciones y mayor mortalidad postoperatoria. En

comparación con los hígados no esteatósicos, los hígados esteatósicos muestran fallo en la regeneración y reducción de la tolerancia frente al daño hepático. De hecho, se ha descrito la existencia de fallo hepático postquirúrgico después de resecciones hepáticas en hígados con esteatosis (Belghiti J., y cols. 2000; Behrns K.E., y cols. 1998). Además, el hecho de utilizar estos hígados en transplantes hepáticos está asociado a un aumento en el riesgo de disfunción hepática después de la cirugía (Ploeg R.J., y cols. 1993; Hayashi M., y cols. 1999). Sin embargo, en muchos casos es necesario recurrir a estos injertos debido a que la lista de espera para trasplante va aumentando, y no puede cubrirse la demanda con hígados en estado óptimo. Además, la esteatosis hepática agrava el problema de la falta de órganos ya que se sabe que entre todos los hígados que no son aptos para trasplante por sus condiciones patológicas, más del 50% son hígados esteatósicos, de ahí que la esteatosis sea la causa del mayor número de órganos no aptos para trasplante, acentuando así la problemática del banco de órganos (Casillas-Ramírez A., y cols. 2006a; Selzner M., y cols. 2001). Dado el aumento en la prevalencia de esteatosis que se espera en la población y por lo tanto en la cirugía hepática, es evidente la necesidad de desarrollar estrategias para minimizar los efectos adversos de la lesión por I/R en los hígados esteatósicos y el fallo en la regeneración hepática que presentan tales hígados. Esto disminuiría por lo tanto el riesgo de disfunción o fallo primario tras la cirugía hepática y se aumentaría el número de injertos disponibles para ser trasplantados. Para lograrlo, es imprescindible el estudio de los mecanismos que conducen a la mayor susceptibilidad de un hígado esteatósico frente a la lesión por I/R y al fallo en la regeneración.

1.5.3.- Lesión por I/R en el hígado esteatósicos

Como se ha mencionado anteriormente, los hígados esteatósicos toleran peor la lesión por I/R que los hígados no-esteatósicos. Sin embargo, las causas de la mayor susceptibilidad de los hígados esteatósicos frente a la lesión por I/R no están totalmente definidas. La literatura recoge como posibles causas los eventos que se describen a continuación.

1.5.3.1.- Alteraciones en la microcirculación

Las alteraciones en la microcirculación se han propuesto como un factor importante en la poca tolerancia que presentan los hígados grasos frente a la lesión inducida por I/R. La acumulación de grasa en el citoplasma de los hepatocitos está asociada con un

aumento en el volumen celular, que puede resultar en una obstrucción parcial o total del espacio sinusoidal hepático (Ricci C., y cols. 1996). Se ha demostrado, tanto en humanos como en ratas con esteatosis inducida por dieta, que en el hígado graso se produce un descenso de aproximadamente el 50% del flujo sanguíneo sinusoidal respecto a los hígados normales, cosa que puede inducir un estado de hipoxia crónica (Seifalian A.M., y cols. 1998; Ijaz S., y cols. 2003; Teramoto K., y cols. 1993b). Este descenso en el flujo sanguíneo es seguramente secundario al estrechamiento de la luz sinusoidal. La luz sinusoidal se ve además afectada por glóbulos de grasa liberados durante la preservación y la reperfusión del injerto y por microtrombos de fibrina y elementos celulares que están en la sangre después de la reperfusión (Teramoto K., y cols. 1993). De esta forma, el edema celular y la adherencia de leucocitos que ocurren durante una I/R en combinación con la reducción del espacio sinusoidal amplifican los efectos negativos producidos por la I/R.

1.5.3.2.- Estrés oxidativo

Los hígados esteatósicos son más susceptibles que los no esteatósicos a la peroxidación lipídica debido a sus bajas defensas antioxidantes y/o su mayor producción de RLO. Durante la reperfusión la generación mitocondrial de RLO aumenta considerablemente, esto conduce a que las estructuras mitocondriales se vean expuestas al ataque de los RLO generados tanto fuera como dentro de estos orgánulos, conduciendo eventualmente a la disfunción mitocondrial y al fallo en la síntesis de ATP. Uno de los efectos importantes de la producción descontrolada de RLO es la peroxidación lipídica de la membrana y de otros lípidos celulares, dando lugar a alteraciones estructurales y al deterioro funcional de los componentes celulares (Elias-Miró M., y cols. 2012a). Además los productos de la peroxidación lipídica, como por ejemplo el malondialdehído, podrían actuar como atrayentes de neutrófilos e inducir necrosis celular (Day C.P., y cols. 1998; Curzio M., y cols. 1985). La causa de este aumento en la peroxidación lipídica podría ser debido a que en los hígados esteatósicos hay una mayor cantidad de substrato fácilmente oxidable (Letteron P., y cols. 1996). En condiciones basales, los sistemas antioxidantes del hígado son suficientes para contrarrestar la peroxidación lipídica que se produce en el hígado. Sin embargo, en hígados esteatósicos sometidos a I/R, la peroxidación de lípidos se ve muy incrementada y los sistemas antioxidantes del hígado se ven sobresaturados.

Diferentes trabajos se han centrado en prevenir el aumento de estrés oxidativo observado en los hígados esteatósicos. No obstante, los datos obtenidos en los estudios en los que se administraban antioxidantes han sido contradictorios. Algunos de estos estudios en ratas Zucker obesas, modelo bien caracterizado de obesidad, indican que la administración de tocoferol, que tiene propiedades antioxidantes, aumenta la tolerancia del hígado esteatósico a la isquemia caliente. Por otro lado, estudios experimentales en hígados esteatósicos inducidos mediante dieta deficiente en colina-metionina, mostraron que la administración de precursores de glutatión (GSH), como la N-acetilcisteína, pueden ayudar a restaurar la integridad hepatocitaria en los hígados esteatósicos pero sin bloquear los RLO. Además, en hígados esteatósicos inducidos por dieta o por alcohol, se producen RLO insensibles a la SOD y catalasa y que están implicados en la vulnerabilidad de este tipo de hígados a la lesión por I/R (Elias-Miró M., y cols. 2012a; Selzner M., y cols. 2000; Soltys K., y cols. 2001; Nardo B., y cols. 2001; Caraceni P., y cols. 2005).

1.5.3.4.- Acumulación de neutrófilos y activación de las células de Kupffer

El exceso de grasa en un hígado esteatósico produce alteraciones en la fluidez de las membranas debido a la menor presencia de colesterol y de ácidos grasos poliinsaturados (Fukumori T. y cols. 1999). Esta alteración de las membranas endoteliales, junto con el mayor deterioro que sufren las células endoteliales tras la isquemia, podría derivar en un aumento en la y activación de los adhesión neutrófilos en la reperfusión, y por tanto a un aumento de la infiltración de neutrófilos en el tejido hepático (Hui A.M., y cols. 1994, Fukumori T., y cols. 1999; Nakano H., y cols. 1997).

La implicación de los neutrófilos en la mayor vulnerabilidad de los hígados grasos frente a la lesión por I/R está documentada en diversos estudios, especialmente en hígados grasos inducidos por alcohol (Teramoto K., y cols. 1993b). En un modelo experimental de trasplante hepático en ratas Zucker se ha demostrado que el bloqueo de integrinas y selectinas, moléculas implicadas en la adhesión de los neutrófilos al endotelio sinusoidal, redujo la lesión hepática y aumentó la supervivencia de las ratas tras el trasplante hepático (Amersi F., y cols. 2002; Amersi F., y cols. 2003; Fondevila C., y cols. 2005). Por otra parte, en otros modelos de esteatosis hepática los neutrófilos no parecen ser los responsables de la poca tolerancia de los hígados esteatósicos frente a la lesión por I/R (Serafín A., y cols. 2002; Nakano H., y cols. 1997; Yamada S., y cols. 2000). En este sentido, se ha observado la misma acumulación de neutrófilos en hígados

esteatósicos que en no esteatósicos en ratas Zucker sometidas a I/R hepática normotérmica, incluso en modelos de esteatósis hepática inducida por la ingesta de una dieta rica en colesterol (Koneru B., y cols. 1995; Sun C.K., y cols. 2001).

En los hígados grasos, se ha demostrado un aumento en el número y en la actividad fagocítica de las células de Kupffer, con respecto a los hígados no esteatósicos (Teramoto K., y cols. 1993b). Este hecho podría explicar la vulnerabilidad de los hígados grasos a la lesión por I/R, ya que las células de Kupffer son una fuente muy importante de RLO y citoquinas pro-inflamatorias como el TNF-α y la IL-1.

1.5.3.5.- Mecanismo de muerte celular

Se ha de mencionar también, que una característica clave del daño isquémico en el hígado no esteatósico es la apoptosis, una forma activa de muerte celular que requiere energía. La apoptosis permite la eliminación de células dañadas con la mínima respuesta inflamatoria. El porcentaje de hepatocitos apoptóticos después de I/R varía según el daño hepático y la supervivencia de los animales de experimentación (Selzner M., y cols. 2000; Selzner M., y cols. 2001; Selzner M., y cols. 2003; Jaeschke H., y cols. 2003; Hong F., y cols. 2004).

Se ha demostrado que los hígados esteatósicos presentan alteraciones en la vía de señalización apoptótica. Estudios realizados en ratas Zucker han señalado que los hígados esteatósicos sometidos a I/R normotérmica presentan menos apoptosis en comparación con hígados no esteatósicos en las mismas condiciones. En cambio, en tales hígados esteatósicos en estos hígados se observa una necrosis masiva. En la muerte celular por necrosis se produce una rotura inespecífica de los orgánulos celulares y de la membrana citoplasmática, así pues se libera el contenido celular, agravando la lesión inflamatoria hepática (Selzner M., y cols. 2001; Behrns K.E., y cols. 1998; Crowley H., y cols. 2000). En consonancia, estrategias terapéuticas basadas en la inhibición de la apoptosis, que reducen el daño isquémico en hígados no esteatósicos, no son capaces de proteger en presencia de esteatosis (Casillas-Ramírez A., y cols. 2006b; Selzner M., y cols. 2000).

El hecho de que se produzca uno u otro tipo de muerte celular podría estar asociado, entre otros factores, con la disponibilidad de ATP de la célula, ya que como se ha comentado anteriormente la apoptosis es un proceso dependiente de ATP. De este modo, el deterioro del metabolismo energético que presentan los hígados grasos tras la I/R hepática podría explicar el fallo de la apoptosis en este tipo de hígados y el

predominio de la lesión por necrosis como forma de muerte celular (Selner M., y cols. 2001; Behrns K.E., y cols. 1998; Crowley .H, y cols. 2000).

Estas diferencias en los mecanismos de muerte celular y en los mecanismos implicados en la lesión por I/R en hígados esteatósicos con respecto a los hígados no esteatósicos explican las dificultades para prevenir efectivamente la lesión por I/R en la cirugía y trasplante hepático. De esta manera, estrategias terapéuticas que son efectivas en hígados no esteatósicos pueden no serlo en presencia de esteatosis, o la dosis efectiva de los fármacos a administrar puede diferir entre los dos tipos de hígados. Por otro lado, también puede haber fármacos que podrían ser efectivos solamente en hígados esteatósicos.

1.6.- Estrategias terapéuticas para disminuir la lesión por I/R

A pesar de los avances en los tratamientos farmacológicos, y en estrategias de terapia génica que han tenido como objetivo el disminuir la lesión por I/R, los resultados hasta el momento no han sido concluyentes.

1.6.1.- Estrategias farmacológicas

Numerosos estudios experimentales se han centrado tanto en inhibir los efectos nocivos de la isquemia como la respuesta inflamatoria asociada a la reperfusión. Con esta finalidad, se han administrado fármacos como la cloroquina o la clorpromazina para prevenir la degradación de fosfolípidos y las disfunciones mitocondriales durante la isquemia hepática. Para inhibir las acciones de los RLO durante la reperfusión se ha tratado con antioxidantes como tocoferol, glutatión éster (GSH-éster), o alopurinol, y se han administrado también anticuerpos dirigidos al TNF para bloquear las acciones nocivas de esta citoquina. También se han realizado tratamientos con dopamina y ATP-MgCl2 para reducir los desórdenes microcirculatorios. Para bloquear la acumulación de neutrófilos se han utilizado también fármacos como la adenosina, donadores de NO, L-arginina, y anticuerpos anti-ICAM-1 y anti-P-selectina. Sin embargo, ninguno de estos tratamientos ha logrado frenar la lesión por I/R hepática (Casillas-Ramírez A., y cols. 2006b).

Se ha de tener en cuenta las dificultades para atenuar la inflamación asociada a este proceso. Como se ha comentado anteriormente, en la respuesta inflamatoria asociada a la reperfusión están implicados múltiples mediadores y tipos celulares. Además, también deben considerarse las dificultades derivadas de los tratamientos farmacológicos. En este sentido, el GSH-éster no llega al lugar de acción a concentraciones óptimas ni en el momento adecuado, o la administración de anticuerpos anti-TNF, que sólo provoca una inactivación parcial de la proteína. Otro ejemplo son los efectos totalmente opuestos que tienen las pequeñas variaciones en la dosis de donadores de NO. Además, no hay que descartar los posibles efectos secundarios derivados de los fármacos, ya que en el caso de la dopamina, la adenosina y los donadores de NO se han descrito efectos nocivos sistémicos (Casillas-Ramírez A., y cols. 2006b).

Si todas estas complicaciones ocurren en hígados sanos, aún son mayores las complicaciones al modular la lesión por I/R en hígados esteatósicos. Como ya se ha comentado, este tipo de hígados generan más RLO y estos son insensibles a la acción de antioxidantes como la SOD y la catalasa. A título de ejemplo, la administración de un donador de NO en un modelo de trasplante hepático experimental logró reducir el estrés oxidativo en hígados sanos, mientras que en injertos esteatósicos aumentó la vulnerabilidad al daño por I/R. Por otra parte, también podrían existir fármacos que sólo fueran efectivos en hígados esteatósicos. Por ejemplo, en un modelo de I/R asociada al trasplante hepático, se ha evidenciado en hígados esteatósicos un aumento en la expresión de la proteína desacoplante-2 mitocondrial (UCP-2) y una capacidad disminuida de sintetizar ATP en la reperfusión, lo que contribuye a la vulnerabilidad de los hígados esteatósicos al síndrome de I/R. Así pues, fármacos como la cerulenina que actua sobre la UCP2 disminuyendo su expresión, logran aumentar el contenido de ATP en hígados esteatósicos, pero tal estrategia tal vez no tendría ningún efecto en hígados sanos porque no se presenta sobreexpresión de UCP-2. Resultados similares se han obtenido con la carnitina (Casillas-Ramírez A., y cols. 2006b).

1.6.2.- Terapia génica

Los actuales avances en biología molecular proporcionan nuevas oportunidades para reducir el daño hepático por I/R a través del uso de la terapia génica. Para reducir el estrés oxidativo con el objetivo de reducir la lesión por I/R hepática, se han realizado pre-tratamientos basados en la sobreexpresión de SOD o catalasa utilizando como vehículo adenovirus, liposomas o polientilenglicol. Para limitar la acumulación y la activación de neutrófilos, se ha reducido la expresión génica de ICAM-1 utilizando liposomas como vehículo. La apoptosis se ha inhibido con la sobreexpresión del gen BcL-2, utilizando principalmente como vehículo adenovirus. Se han desarrollado estrategias citoprotectoras basadas en la expresión de genes tales como la heme oxigenasa-1 (HO-1), la citoquina anti-inflamatoria IL-13 y el antagonista del receptor de la IL-1 (IL-1ra), empleando como vectores adenovirus o liposomas. También se ha intentado modular la acción del NF-κB a través de transfección adenoviral de una forma mutante de IκB, el cual inhibiría NF-κB y aliviaría la respuesta inflamatoria hepática asociada a la I/R. A pesar de estas aproximaciones farmacológicas, aún existen graves problemas entorno a la terapia génica, como son la toxicidad de los vectores, la dificultad en conseguir una expresión óptima de la proteína en el momento y lugar

adecuado, y la dificultad de conseguir mutantes adecuados (en el caso de NF-κB), debido a las controversias existentes en la activación de NF-κB (Casillas-Ramírez A., y cols. 2006b).

En los últimos años, el uso de las terapias génicas basadas en RNA interferentes pequeños (siRNA) para regular las acciones biológicas de los mediadores inflamatorios en una gran variedad de enfermedades (Ichim T.E., y cols. 2004) ha despertado gran interés. Así pues, se han aplicado estrategias terapéuticas basadas en la administración de siRNA para adiponectina (Massip-Salcedo M., y cols. 2008) esfingomielinasa acídica (Llacuna L., y cols. 2006), o moléculas relacionadas con la apoptosis tales como Bax (Sass G, y cols. 2007), el receptor apoptótico Fas (Li X., y cols. 2007), la Caspasa 8 y la Caspasa 3 (Contreras J.L., y cols. 2004); las cuales han protegido frente al daño hepático inducido por I/R. Sin embargo, de la misma manera que pasaba con los adenovirus, los liposomas o el polientilenglicol, uno de las mayores complicaciones para que estas estrategias puedan alcanzar su aplicación clínica es la eliminación de sus efectos secundarios mediante, en parte, el desarrollo de estrategias que sean capaces de permitir un silenciamiento génico estable. Dado que los siRNA son ácidos nucleicos, se degradan fácilmente en suero y por lo tanto necesitan un vehículo que permita un transporte estable, una internalización celular específica y una farmacocinética favorable para utilizarse como terapia farmacológica. El riesgo de inmunogenicidad así como la posible toxicidad de estos vehículos, también son obstáculos que deben ser superados antes de poder aplicarse en la práctica clínica (Pellish R.S., y cols. 2008).

1.6.3.- Estrategias quirúrgicas

Diversas estrategias quirúrgicas han demostrado ser protectoras frente a la lesión por I/R hepática, tales como el shunt portosistémico, el clampaje intermitente o el precondicionamiento isquémico (PC). Los éxitos de nuestro grupo respecto a la eficacia del PC en hígados esteatósicos y no esteatósicos sometidos a isquemia caliente asociada a hepatectomía parcial y a trasplante hepático se han traducido en la aplicación clínica del PC (Elias-Miró M., y cols. 2012d).

A partir del momento en que se describió la efectividad del PC, se han realizado numerosos trabajos con la finalidad de buscar estrategias que puedan mimetizar sus efectos beneficiosos. Una de estas estrategias es el "heat shock", que consiste en inducir un aumento en la temperatura corporal antes de la isquemia hepática. También se ha intentado realizar un pre-condicionamiento químico con dexorrubicina, factor

natriurético atrial, o con oxidantes, y se ha demostrado que estos tratamientos reducen la lesión hepática en diferentes modelos experimentales de I/R. Sin embargo, las limitaciones de estas estrategias es su posible aplicación clínica, bien por la dificultad que ello supondría, por problemas de toxicidad o por los efectos secundarios descritos (Casillas-Ramírez A., y cols. 2006b).

Las investigaciones acerca de la efectividad del PC en modelos experimentales de I/R hepática han sido la base para que esta estrategia quirúrgica sea la única que ha alcanzado su proyección en la práctica clínica para reducir la lesión por I/R hepática normotérmica asociada con las resecciones hepáticas de tumores, tanto en hígados sanos como en esteatósicos. Varios estudios han demostrado la eficacia del PC en la resección de los hígados esteatósicos y no esteatósicos en la práctica clínica. En estos estudios, los autores realizaron principalmente resección hepática a través de una maniobra de Pringle continua. Sin embargo, otros datos indican que el PC no mejora la función hepática postoperatoria y no afecta la morbilidad o la mortalidad después de la hepatectomía bajo exclusión vascular del hígado con la preservación del flujo de la vena cava. Las discrepancias entre estos efectos diferenciales del PC durante la resección hepática podrían haber surgido a partir de la ausencia de flujo de perfusión del hígado durante la exclusión vascular en comparación con la maniobra de Pringle. Además, el período de isquemia utilizado en ambos estudios (Clavien P.A., y cols 2003; Azoulay Z., y cols 2006) fue diferente. Todo esto podría explicar, al menos parcialmente, la diferente eficacia del PC en la práctica clínica de la cirugía del hígado (Elias-Miró M., y cols. 2012d).

En la última década, se han hecho grandes esfuerzos para trasladar los efectos beneficiosos del PC a la práctica clínica del trasplante hepático. Los resultados clínicos en general han sido menos evidentes que las observaciones en animales de experimentación. Sin embargo, estos efectos diferenciales no pueden ser explicados por el uso de períodos de PC en la clínica que han resultado ser ineficaces experimentalmente o por el uso clínico de diferentes tiempos de isquemia fría a los evaluados experimentalmente. Sin embargo, se debe considerar el número limitado de pacientes con esteatosis incluidos en los ensayos clínicos de PC y la presencia de muerte cerebral en el trasplante hepático clínico, la cual no se ha tenido en cuenta en los estudios experimentales realizados hasta el momento (Elias-Miró M., y cols. 2012d).

Por consiguiente, en el futuro, los ensayos clínicos deberían incluir una mayor proporción de donantes con hígados esteatósicos para aclarar la eficacia del PC en el

trasplante hepático en la práctica clínica. Los beneficios del PC son más clínicamente significativos en los grupos de pacientes con un mayor riesgo de morbilidad y mortalidad, es decir, en los pacientes con esteatosis hepática y cirrosis. De hecho, en el estudio prospectivo y aleatorio de PC en hepatectomía parcial, se demostró que el PC es más eficaz en la reducción de la lesión por reperfusión en pacientes con hígados esteatósicos. En otro estudio se informó que el PC redujo el riesgo de insuficiencia hepática y acortaba la estancia hospitalaria de pacientes con cirrosis sometidos a hepatectomía parcial. Existe la posibilidad de que el PC no sea eficaz en el contexto de la muerte cerebral, ya que los donantes de órganos fallecidos tienen inestabilidad hemodinámica con una disminución media de la presión arterial, y del flujo sanguíneo venoso portal y del tejido hepático. Además, la muerte cerebral induce una intensa respuesta inflamatoria sistémica que se manifiesta en muchos órganos, incluyendo el hígado. Es muy probable que en el marco de tales respuestas inflamatorias los beneficios del PC puedan no ser evidentes. No existen estudios de PC en trasplante hepático en presencia de muerte cerebral. Tales investigaciones experimentales podrían abordar un problema clínico importante en el trasplante hepático, ya que más del 80% de los hígados utilizados son de donantes cadavéricos, y solo el 20% de estos tienen una esteatosis hepática leve o moderada.

1.7.- La proteína transportadora de retinol tipo 4 (RBP4) y el retinol

1.7.1.- Características del RBP4

El término "adipoquina" o "adipocitoquinas" comprende a polipéptidos, los cuales son producidos mayoritariamente en el tejido adiposo. Además de los adipocitos, el tejido adiposo está compuesto del estroma que incluye macrófagos, fibroblastos y monocitos que se han infiltrado, los cuales contribuyen a la producción de adipocitoquinas. En los últimos años, las investigaciones se han centrado en las adipocitoquinas como potenciales dianas terapéuticas en distintas patologías relacionadas con la obesidad y el síndrome metabólico. Desde hace tiempo se conoce que regulan la esteatosis, la inflamación y la fibrosis (Marra F., y cols. 2009). Estudios recientes han implicado las adipocitoquinas en la vulnerabilidad de los hígados esteatósicos a la lesión por I/R (Elias-Miró M., y cols. 2011; Massip-Salcedo M., y cols. 2008; Man K., y cols. 2006; Casillas-Ramírez A., y cols. 2011; Elias-Miró M., y cols. J Hepatol 2014). Esto derivaría en el desarrollo de nuevas estrategias terapéuticas de utilidad para reducir la lesión por I/R de hígados esteatósicos.

El RBP4 es una adipocitoquina sintetizada en el hígado, cuya función conocida es transportar retinol en la circulación. Sin embargo, el papel del RBP4 en el hígado es en gran parte desconocido (Blane W.S., 1989; Graham T.E., y cols. 2006; Wagnerberger S., y cols. 2006). Desde el descubrimiento del RBP4 en 1992, diversos estudios han demostrado que los niveles de RBP4 están elevados en diabetes, obesidad, enfermedades cardiovasculares e inflamación (Graham T.E., y cols. 2006; Yang Q., y cols. 2005; Cho Y.M., y cols. 2006; Lee D.C., y cols. 2007; Yao-Borengasser A., y cols. 2007). Por el contrario, se ha observado que en injertos esteatósicos sometidos a un transplante con I/R los niveles de RBP4 decrecían. Existe una correlación positiva entre altos niveles circulantes de RBP4 e infiltración grasa en hígado, tanto en modelos experimentales como en humanos (Yang Q., y cols. 2005; Seo J.A., y cols. 2008; Wu H., y cols. 2008). Debido a que el hígado es la principal fuente generadora de RBP4, cuando este sufre un daño se detectan elevados niveles circulantes de RBP4 (Wu H., y cols. 2008).

Diversos trabajos en la literatura se han centrado en investigar los mecanismos de acción del RBP4 en diferentes patologías. Se ha descrito que el RBP4 por sí mismo

puede ejercer efectos pro-inflamatorios a través de alterar la producción de otras adipocitoquinas o citoquinas inflamatorias (Graham T.E., y cols. 2006; Yao-Borengasser A., y cols. 2007; Balagopal P., y cols. 2007; Wu H., y cols. 2008). A título de ejemplo, aumentos en los niveles plasmáticos de RBP4 observados en pacientes obesos, están asociados con aumentos en marcadores inflamatorios como el TNF y MCP-1 (Graham T.E., y cols. 2006). Además el RBP4 afecta la generación de adiponectina en pacientes obesos y en determinadas patologías como la diabetes (Wu H., y cols. 2008). Notoriamente, el TNF, MCP-1 y la adiponectina son mediadores implicados en la lesión por I/R hepática (Casillas-Ramírez A., y cols. 2006b).

Teniendo en cuenta estas observaciones, se puede considerar la posibilidad de que los hígados esteatósicos al ser sometidos a una hepatectomía parcial con I/R tengan menores niveles de RBP4. Hasta la fecha, no se ha estudiado si una inhibición o administración de RBP4 puede proteger a los hígados esteatósicos frente a la lesión inducida por I/R normotérmica.

1.7.2.- RBP4 en I/R hepática

Existen diferentes trabajos encaminados a modular farmacológicamente la acción del RBP4. La administración de fenretinida ha sido efectiva como tratamiento en diabetes de tipo 2 ya que ha logrado reducir los niveles plasmáticos de RBP4 (Yang., y cols. 2005). Otra posibilidad farmacológica para bloquear la acción del RBP4 consistiría en la administración de anticuerpos anti-RBP4 o en terapias basadas en interferencia del RNA del RBP4. Los anticuerpos anti-RBP4 se han utilizado tanto *in vitro*, (Ost., y cols. 2007) como *in vivo* (Casillas-Ramírez A., y cols. 2011). En este último, la administración de RBP4, o la inducción de esta adipocitoquina mediante precondicionamiento isquémico, tiene efectos beneficiosos en el daño hepático por I/R en un modelo de transplante hepático con injertos esteatósicos (Casillas-Ramírez A., y cols. 2011). Por el otro lado, no hay estudios acerca de la eficacia del tratamiento co.n siRNA de RBP4 *in vivo*, pero podría tener interés teniendo en cuenta que estas terapias génicas basadas en la interferencia del RNA pueden cumplir criterios necesarios para su posible utilización clínica, tales como especificidad en los efectos de silenciamiento y eficacia in vivo (Ichim T.E., y cols. 2004). Por otra parte, se tendría que evaluar la transfección génica de estos siRNA de RBP4 ya que en muchos casos cuando se utiliza una terapia génica, (Massip-Salcedo M., y cols. 2008; Llacuna L., y cols. 2006; Sass G., y cols. 2007; Li X., y cols. 2007; Contreras J.L., y cols. 2004) se ha de pre-tratar al

donante al menos 24 horas antes de su extracción e implantación en el receptor para conseguir una transfección génica eficaz en el injerto, lo cual tiene sus limitaciones en la práctica clínica.

1.7.3.- RBP4 en la esteatosis hepática

Numerosos estudios sugieren una correlación de los niveles de RBP4 con un aumento de la adiposidad, hasta se ha implicado el RBP4 en el enfermedad del hígado graso no alcohólico (NAFLD). Mientras Huang S.H. y Yang Y.J. encontraron que altos niveles de RBP4 son independientes de NALFD pero un valor indicativo válido de la hipertrigliceridemia en niños; Nobili y cols., encontraron que los niveles de RBP4 eran inversamente correlacionados con la gravedad histológica de NAFLD, por lo contrario, Huang S.C. y cols., encontraron una correlación positiva entre los niveles de RBP4 y la gravedad de NAFLD (Nobili V., y cols 2009; Huang S.C., y cols. 2013). Estas diferencias fueron atribuidas a una selección de la población con distinta severidad y duración de la enfermedad hepática. Se concluyó que el RBP4 podría comportarse de manera diferente según el estadio de NAFLD del paciente (Huang S.C., y cols. 2013). Por otro lado, en los modelos experimentales de ratones ob/ob y ratones tratados con una dieta rica en grasas, se observaba una sobreregulación de RBP4 en el tejido adiposo. En línea con esto, otros estudios también han demostrado que el RBP4 está relacionado con elevados niveles de grasa hepática y daños en el hígado, lo que indica que el RBP4 podría ser un marcador circulatorio de NAFLD (Tan Y., y cols. 2011). En otro estudio dónde el RBP4 se ha relacionado con esteatosis hepática, el tratamiento de ratones ob/ob con fenretinide reguló el grado de hígado grasos, al menos en parte, por la reducción de los niveles de RBP4 plasmático.

1.7.4.- El retinol y los ésteres de retinilo, y su relación con el RBP4

El retinol o vitamina A, es una vitamina liposoluble, esencial para el ser humano y que es introducida en el cuerpo mediante la alimentación. Bajo condiciones normales, la mayoría de la vitamina A es almacenada en el hígado, principalmente en las células estrelladas. Las células estrelladas almacenan el 80% del retinol hepático y lo liberan según el estatus extracelular del retinol. Aunque el paso del retinol de unas células a otras aún no se conoce, si se sabe que es almacenado en forma de ésteres de retinilo en hepatocitos o en gotas lipídicas en las células estrelladas (Bellovino D., y cols. 1984; Tuitoek P.J., y cols. 1996; Lee Y.S., y cols. 2012). La movilización de estos ésteres de

retinilo requiere su hidrólisis a retinol libre, y este es liberado a la circulación junto con el RBP4 (peso molecular de 21 000 Da), formando el complejo retinol-RBP4. El complejo retinol-RBP4 (holo-RBP4) es secretado a la circulación, dónde se une con la transtiretina (TTR), una proteína transportadora de tirosina (peso molecular de 55 000 Da) (Tuitoek P.J., y cols. 1996). Están unión de la TTR al holo-RBP4 estabiliza el complejo en la circulación. Cuando el retinol es entregado a las células dianas, el retinol cruza la membrana plasmática por difusión simple o a través de un receptor específico para RBP4. Este mecanismo aun no se conoce con exactitud. Una vez separado del retinol, el RBP4 pierde su afinidad por el TTR, vuelve a la circulación como apo-RBP4 (sinR) y es eliminado a través de los riñones (**Figura 10**). En las células dianas el retinol es metabolizado a ácido retinoico y se une a los receptores nucleares RARs-RXRs para promover la transcripción (Bellovino D., y cols. 1984; Tuitoek P.J., y cols. 1996; Lee Y.S., y cols. 2012).

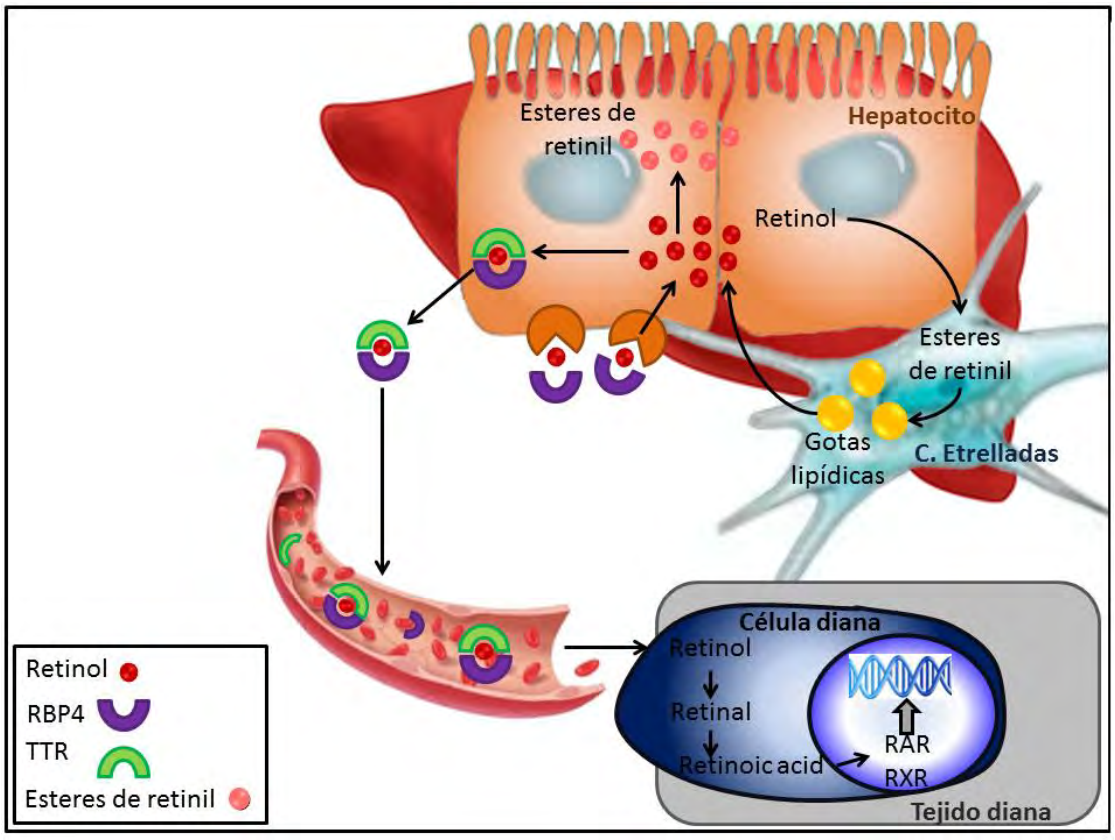


Figura 10. Dibujo esquemático del transporte y almacenaje del retinol.

Desde hace años se ha demostrado que la secreción hepatocítica del RBP4 es regulada por el retinol. De hecho, como se muestra en el hígado de ratas alimentadas con dietas sin vitamina A, la liberación del RBP4 es inhibida y se acumula en el hígado en

ausencia de vitamina A. Al administrar vitamina A, este RBP4 es inmediatamente secretado a la circulación (Ronne H., y cols. 1983; Tuitoek P.J., y cols. 1996).

1.7.5.- Retinol en I/R hepática

Como se comentará en el siguiente apartado de la presente tesis, los niveles de vitamina A, un potente antioxidante, en pacientes con enfermedad hepática crónica, están reducidos en comparación con pacientes sanos. Tras la reperfusión de injertos esteatósicos, los niveles de vitamina A se reducen, sobre todo en los pacientes con enfermedad hepática crónica. Estos pacientes tienen disminuidas sus defensas antioxidantes, y esto provoca una mayor lesión hepática por I/R. De esta manera, terapias antioxidantes como sería el pre-tratamiento con retinol podría mejorar los efectos de la I/R en estos pacientes (Goode H.G., Hepatology 1994). Es más, se ha demostrado que el ácido al-trans-retinoico protege los hígados no esteatósicos contra los efectos dañinos del estrés oxidativo en isquemia caliente sin hepatectomía (Rao J., y cols. 2010). Más tarde, este mismo grupo demostró que la administración de al-trans-retinoic también protegía frente al daño inflamatorio inducido por la isquemia caliente mediante la inhibición de NF-κB (Rao J., y cols. 2013).

1.7.6.- Retinol en la esteatosis y regeneración hepáticas

Como se ha comentado anteriormente, en condiciones fisiológicamente normales, las células estrelladas almacenan el 80% del retinol corporal en las gotas lipídicas de su citoplasma, mostrando heterogeneidad zonal según su capacidad de almacenar retinol. El tamaño y el número de estas gotas lipídicas están marcadamente influenciado por la ingesta de retinol, y por su administración. Se ha observado que tras una hepatectomía parcial del 70%, las gotas lipídicas de estas células disminuían durante los 3 días siguientes a la hepatectomía, y no se recuperaban hasta 14 días después. Incluso desaparece la heterogeneidad de las áreas de almacenaje del retinol de las células estrelladas tras la hepatectomía parcial, y no regresaba a la normalidad hasta 14 días después de la cirugía, aun y cuando el hígado había recuperado su volumen inicial. En la formación de estas gotas lipídicas el enzima lecitin:retinol acetiltransferasa (LRAT) juega un papel fundamental. Hasta el punto que las células estrelladas de ratones *Lrat*$^{-/-}$ no contienen gotas lipídicas, y esto se ve acompañado por una ausencia de ésteres de retinilo en estos hígados. Utilizando este mismo modelos de ratones *Lrat*$^{-/-}$, Shmarakov I.O. y cols. demostraron que estos ratones tenían menores niveles de ácido all-trans-

retinoic, y esto provocaba un retraso en la regeneración hepática tras una hepatectomía parcial (Shmarakov I.O., y cols. 2013).

Por otro lado, se han observado altos niveles de vitamina A en pacientes con esteatosis simple y en pacientes con NASH. En este mismo estudio, los niveles plasmáticos de vitamina A estaban relacionados con la severidad histopatológica del NASH y los niveles de vitamina A eran mayores cuanto más infiltración lipídica se encontraba en el hígado (Bahcecioglu I.H., 2005). En otro estudio, donde se pretendía estudiar la función biológica del ácido retinoico, se utilizaron ratones transgénicos expresando RARE, la forma negativa de RARα, que puede suprimir las actividades endógenas de los heterodímeros RAR/RXR. Se observó que estos ratones desarrollaban esteatohepatitis grave. De este modo se evidencia que el ácido retinoico es indispensable para el desarrollo normal de la arquitectura hepática, y que perdida de la funcionalidad del ácido retinoico y derivados conduce a la aparición de esteatosis severa, entre otras patologías. En este mismo estudio observaron que estos ratones que sobreexpresan RARE, tenían un aumento en la actividad de la β-oxidación, sobretodo de la β-oxidación peroxisomal. Esto en parte era debido a una sobrer-regulación del PPAR-α (Yanagitani A., 2004). Es más, el agonista del PPAR-α protege a los hígados esteatósicos sometidos a I/R normotérmica, pero no a hígados no esteatósicos (Massip-Salcedo M., y cols. 2007).

1.8.- Receptor activador de la producción de peroxisomas (PPAR)

1.8.1.- Características del PPAR

Los peroxisomas son orgánulos subcelulares del hepatocito que contienen una batería de enzimas antioxidantes que protegen los hepatocitos del daño por oxidantes. La proliferación de los peroxisomas en los hepatocitos es inducida, al menos en parte, por la activación de los PPAR (Okaya T., y cols. 2004). Los PPAR pertenecen a la superfamília de receptores nucleares hormonales, y existen tres isoformas:

- PPAR-α
- PPAR-γ
- PPAR-β/δ

De todos ellos, se ha demostrado que sólo los PPAR-α y PPAR-γ son importantes reguladores del daño post-isquémico, ejerciendo sus efectos sobre la esteatosis y la inflamación hepática (Elias-Miró M., y cols. 2012d).

Se ha demostrado que la presencia de grasa en el hígado no induce cambios en los niveles de PPAR-α y PPAR-γ. Aun y así hay controversias. Mientras Massip-Salcedo, y cols., y Casillas-Ramírez, y cols., no observaron cambios al comparar ratas Zucker esteatósicas con no-esteatósicas, Inoue y cols, y Zhao y cols, observaron mayores y menores niveles de PPAR-γ respectivamente en los hígados esteatósicos y no esteatósicos, respectivamente. Estas diferencias se podrían explicar en parte por los distintos modelos experimentales usados. Por otro lado, se han observado diferentes niveles de PPAR-α según el tipo de hígado. En este sentido los hígados esteatósicos sometidos a isquemia caliente y los hígados de pacientes obesos con NAFLD presentan niveles de PPAR-α más bajos que los hígados no esteatósicos (Elias-Miró M., y cols. 2012d).

Los PPARS pueden activar o inhibir la expresión génica, mediante dos mecanismos, la transactivación y la transrepresión (**Figura 11**).

- En la transactivacion, dependiente de DNA y de ligando, los PPAR activan la transcripción uniéndose directamente a elementos específicos de respuesta PPAR

(PPREs) en los genes diana como el RXR. La unión del agonista conduce al reclutamiento del complejo co-activador que modifica la estructura de la cromatina, facilitando el montaje de la maquinaria transcripcional en el promotor.

- La transrepresión, dependiente de ligando, podría explicar las acciones anti-inflamatorias del PPAR. Los PPAR reprimen la transcripción antagonizando las acciones de otros factores de transcripción.

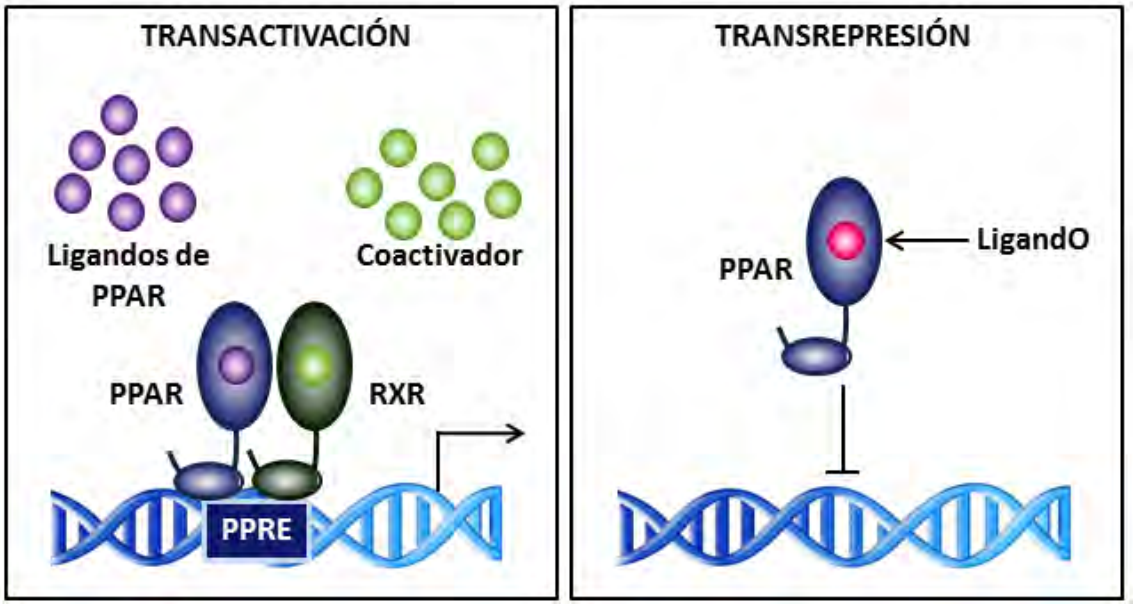


Figura 11. Mecanismos de acción del PPAR.

Fisiológicamente, los heterodímeros PPAR-RXRs deben unirse a los PPREs en ausencia de un ligando. Aunque la activación transcripcional depende de la unión entre el heterodímero PPAR-RXR con el ligando, la presencia de PPAR-RXR sin ligando tiene efectos que varían en función del promotor y del tipo de célula. Futuras investigaciones sobre la estructura de los PPAR y sus mecanismos de regulación transcripcional pueden ser útiles para el diseño de nuevas estrategias terapéuticas. Como se explicará en los siguientes apartados, las estrategias farmacológicas utilizadas actualmente y que se basan en la regulación de PPAR, no podrían incorporarse en la cirugía del hígado debido a sus potenciales efectos secundarios. Aun y así, teniendo en cuentas las propiedades anti-inflamatorias y anti-obesidad de PPAR-α y PPAR-γ, estrategias farmacológicas dirigidas a estos factores de transcripción podrían ser prometedoras contra la esteatosis hepática en pacientes sometidos a I/R. Sin embargo, es importante tener en cuenta que estas estrategias farmacológicas dependerán del tipo de isquemia

(fría o caliente), de la duración de esta, y del tipo de hígado (esteatósicos o no esteatósicos) (Elias-Miró M., y cols. 2012d).

1.8.2.- PPAR en I/R hepática

Pocos estudios han examinado tanto la expresión de PPAR-α hepática inducida por I/R, como los beneficios potenciales de antagonistas de PPAR-α en estas condiciones. De acuerdo con estudios previos de nuestro grupo, los niveles proteicos y de mRNA de PPAR en hígado no esteatósicos durante la I/R fueron similares a los del grupo sham (no operado), y no jugaba un papel crucial en la lesión por I/R en hígados no esteatósicos (Massip-Salcedo M., y cols. 2008). Esto contrastaba con estudios publicados por Okaya y Lentsh (Okaya T., y cols. 2004) y Xu y cols. (Xu S.Q., y cols. 2008), que informaron de los beneficios de los agonistas de PPAR-α en el daño post-isquémico. Los efectos protectores fueron posiblemente asociados con reducciones en la acumulación de neutrófilos, el estrés oxidativo, y la expresión de TNF y IL-1 (**Figura 12**). Aunque la dosis y el tiempo de tratamiento con el agonista de PPAR-α Wy-14,643 fueron similares, Okaya y Lentsh (Okaya T., y cols. 2004) y Xu y cols. (Xu S.Q., y cols. 2008) usaron un tiempo de isquemia de 90 minutos, mientras el tiempo de isquemia de nuestro grupo fue de 60 minutos. Por lo tanto, 60 minutos de isquemia parecen insuficientes para inducir cambios en los niveles de PPAR-α en los hígados no esteatósicos. En la esteatosis simple, el tratamiento de ratones con el agonista Wy-14,643 protege los hígados esteatósicos frente la lesión por I/R, y los beneficios de este tratamiento potencialmente ocurren a través de la amortiguación de la molécula de adhesión y esteatohepatítis no alcohólica (NASH las respuestas de citoquinas, la activación de NF-κB y la producción de IL-6. En hígados esteatósicos sometidos a isquemia caliente, estrategias capaces de aumentar el PPAR-α tales como la administración de agonistar de PPAR-α o inductores de PC, inhiben laexpresión de MAPKS (**Figura 12**). Esto a su vez inhibe la acumulación de adiponectina en los hígados esteatósicos, y los efectos dañinos de la adiponectina sobre el estrés oxidativo y la lesión hepática. Teniendo en cuenta estos datos, la regulación del PPAR-α podría ser un método alternativo para la reducción del estrés oxidativo presente en los hígados esteatósicos tras la I/R. Esto evitaría la necesidad de utilizar terapias frente al estrés oxidativo. En un estudio reciente de hígados no esteatósicos sometidos a isquemia hepática caliente, la suplementación con ácidos grasos n-3 poliinsaturados (PUFA) aumentó los niveles de n-3 PUFA hepáticos y redujo los niveles hepáticos de n-6/n-3

PUFA. Esto se asoció con una sobrerregulación de PPAR-α, que a su vez redujo la señalización de NF-κB y el estrés oxidativo, disminuyendo la respuesta inflamatoria (Elias-Miró M., y cols. 2012d).

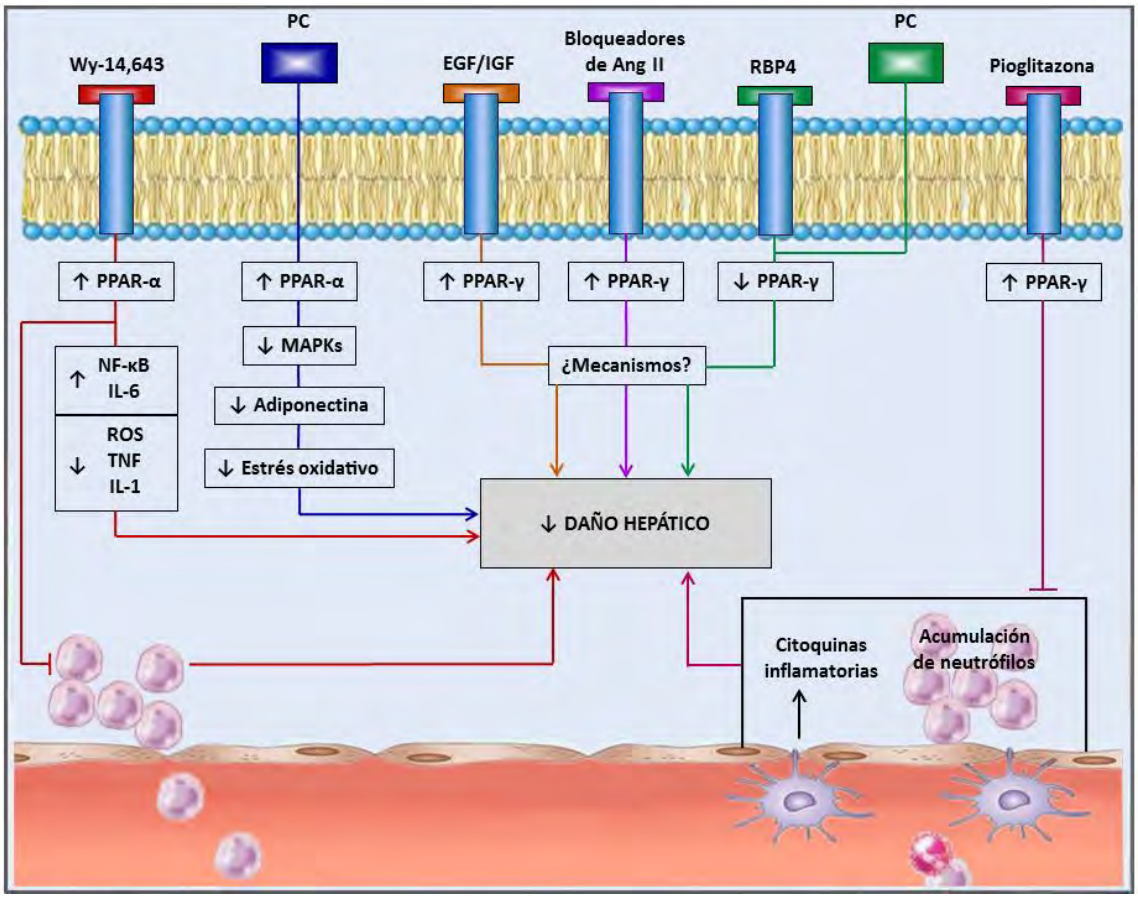


Figura 12. PPAR en I/R hepática.

La función del PPAR-γ en la lesión hepática por I/R no está clara. Resultados previos en estudios de trasplante hepático indican que la I/R no indujo cambios en la expresión de PPAR-γ en los hígados no esteatósicos, y, en consecuencia, estrategias basadas en su regulación no tuvieron ningún efecto sobre la lesión hepática. Estos resultados fueron diferentes de los observados en los hígados no esteatósicos en condiciones de isquemia caliente. En este estudio, el tratamiento con pioglitazone, un agonista de PPAR-γ, inhibió significativamente la lesión hepática por I/R (**Figura 12**). Este efecto protector fue asociado con la inhibición de varias citocinas pro-inflamatorias y la acumulación de neutrófilos. Estos resultados estan en consonancia con otros datos que indican que los ratones deficientes en PPAR-γ muestran lesiones más graves que ratones no tratados en condiciones de isquemia caliente. Por otra parte, el tratamiento con pioglitazone inhibió la apoptosis y mejoró significativamente la supervivencia de los ratones en un modelo

letal de I/R hepática. Estudios previos indicaron que la activación de PPAR-γ inhibe la liberación de TNF-α, IL-1 e IL-6 por los macrófagos, lo que podría ser de interés en los hígados esteatósicos. En efecto, en condiciones de I/R normotérmica, se detectaron niveles más altos de IL-1 y más bajos de IL-10 en hígados esteatósicos que en hígados no esteatósicos. Este desequilibrio entre interleuquinas pro inflamatorias y anti-inflamatorias aumentó el estrés oxidativo y disminuyó la tolerancia de los hígados esteatósicos a la lesión por I/R (Elias-Miró M., y cols. 2012d).

Los resultados anteriores indican que la activación de PPAR-γ con rosiglitazone aumenta la autofagia y protege contra la lesión hepática por I/R. La autofagia es el proceso de reciclaje de proteínas y orgánulos viejos a través de la degradación lisosomal. Por lo tanto, estos resultados sugieren que el PPAR-γ tiene propiedades anti-inflamatorias y puede ser relevante durante la lesión hepática de I/R. De acuerdo con estos datos, la activación de PPAR-γ sería un mecanismo clave en estrategias farmacológicas o quirúrgicas para hígados esteatósicos sometidos a I/R. En línea con esto, resultados basados en hígados perfundidos aislados indicaron que la adición de factores de crecimiento [factor de crecimiento epidérmico (EGF) y de crecimiento similar a la insulina factor de - 1 (IGF-I)] a la solución de preservación de la Universidad de Wisconsin (UW) protegía los hígados esteatósicos debido a la sobreexpresión de PPAR. Del mismo modo, pre tratamientos con EGF protegieron los hígados esteatósicos sometidos a isquemia caliente mediante la sobreexpresión de PPAR-γ (**Figura 12**). Por otra parte, en isquemia hepática caliente, una regulación positiva del PPAR-γ era un mecanismo clave en los beneficios de los bloqueadores farmacológicos de la angiotensina II [antagonistas de la angiotensina II e inhibidores de la enzima convertidora de angiotensina (ACE)] en hígados esteatósicos. Sin embargo, tal y como se ha comentado anteriormente, el papel del PPAR-γ en la lesión hepática por I/R podría depender de las condiciones quirúrgicas, como ha demostrado un reciente estudio en modelo de trasplante hepático dónde un antagonista del PPAR-γ fue eficaz en los hígados esteatósicos, sugiriendo un papel perjudicial del PPAR-γ en estas condiciones. En consonancia con este hallazgo, la inhibición del PPAR-γ era un mecanismo clave en los beneficios del tratamiento con RBP4 y PC en injertos hepáticos esteatósicos. Teniendo en cuentas estos resultados, fármacos dirigidos a la regulación del PPAR-γ pueden potencialmente aumentar el número de órganos aptos para el trasplante. Sin embargo, los datos reportados sobre PPAR-γ en modelos de trasplante de hígados esteatósicos no deben extrapolarse a los trasplantes de injertos esteatósicos de

tamaño reducido. En tal situación clínica, se debe considerar la regeneración del hígado inherente a este procedimiento quirúrgico y el mecanismo de daño hepático derivado ed la eliminación de la masa hepática (Elias-Miró M., y cols. 2012d).

1.8.3.- PPAR en la esteatosis hepática

Numerosos estudios sugieren que las acciones del PPAR-α pueden prevenir la esteatosis. Los ratones deficientes en PPAR-α desarrollan esteatosis hepática tanto en ayunas como al alimentarse con una dieta altamente rica en grasas. En línea con estos resultados, el tratamiento con un agonista de PPAR-α disminuyó la esteatosis en ratones alimentados con una dieta deficiente en metionina y colina (MCD). En otro estudio, la activación de PPAR-α con el agonista Wy-14,643 mejoró el NASH y la esteatohepatítis inducidas con una dieta MCD. El papel crítico de los PPAR-α en la mejora de la esteatosis está mediado a través de la regulación de una amplia variedad de genes implicados en los sistemas de β-oxidación peroxisomal, mitocondrial, y microsomal de ácidos grasos en el hígado. Cuando los hígados esteatósicos son sometidos a ciertos tipos de estrés, como la hepatectomía parcial, la activación del PPAR-α por el benzafibrato reduce la disponibilidad de ácidos grasos de la circulación, reduciendo de este modo la síntesis de esfingolípidos hepáticos (**Tabla 2**) (Elias-Miró M., y cols. 2012d).

Es bien conocido que el n-3 PUFA y sus ácidos grasos derivados activan el PPAR-α, que luego se heterodimeriza con RXR y el receptor X del hígado, dando lugar a la transcripción de un gran número de genes implicados en el metabolismo de los lípidos. Los n-3 PUFA son activadores de PPAR-α *in vivo* más potentes que los n-6 PUFA. Además, los metabolitos de los PUFA, tales como los eicosanoides o los ácidos grasos oxidados tienen uno o dos veces más afinidad por PPAR-α, y son consecuentemente activadores transcripcionales de genes dependientes PPAR-α (Elias-Miró M., y cols. 2012d).

PPARα PPARα activators					
Strategies	Time	Effect	Experimental model and patients	Steatosis and hepatic injury	Regeneration
WY-14,643 (30 μmol/kg/d) [17]	3 weeks	↑ PPARα	Obese Zucker rats	↑ β-oxidation of fatty acids	Not evaluated
WY-14,643 (180 μmol/kg/d) [17]	1 week	↑ PPARα	Ob/ob mice	↑ β-oxidation of fatty acids; ↓ triglycerides	Not evaluated
WY-14,643 (10 mg/kg) [23, 24]	1 h before ischemia	↑ PPARα	Mice or Rats; warm ischemia (90 min)	↓ hepatic injury	Not evaluated
WY-14,643 (10 mg/kg) [13]	1 h before ischemia	↑ PPARα	Zucker obese rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
WY-14,643 (10 mg/kg) [25]	10 days before surgery	↑ PPARα	Foz/foz mice; steatotic livers; warm ischemia (90 min)	↓ hepatic injury	↑ cell cycle entry
Wy-14,643 (0.1%) [37]	5 weeks	↑ PPARα	Mice fed MCD diet	↓ steatohepatitis	Not evaluated
Wy-14,643 (0.1%) [38]	12 days	↑ PPARα	Mice fed MCD diet	↓ steatohepatitis; ↑ hepatic fatty acid oxidation	Not evaluated
Bezafibrate [39]	5 weeks	↑ PPARα	Mice fed MCD	↓ hepatic triglycerides; ↑ hepatic fatty acid oxidation	Not evaluated
Benzafibrate (75 mg/kg) [40]	7 days	↑ PPARα	Rats; partial hepatectomy	↓ availability of fatty acids; sphingolipid synthesis	↓ liver regeneration
PC (5 min/10 min) [13]	Immediately before ischemia	↑ PPARα	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
n-3 PUFA (EPA (270 mg/kg) and DHA (180 mg/kg)) [28]	7 days	↑ PPARα	Sprague-Dawley rats; warm ischemia	↓ hepatic injury, inflammation, and oxidative stress	Not evaluated
EPA (2700 mg/d) [41]	1 year	↑ PPARα	NAFLD patients	↓ steatosis, hepatic injury, necroinflammation, and oxidative stress	Not evaluated
n-3 PUFA (1 g/day) [42]	1 year	↑ PPARα	NAFLD patients	↓ steatosis, hepatic injury, and necroinflammation	Not evaluated
n-3 PUFA (2 g/day) [43]	6 months	↑ PPARα	NAFLD patients	↓ steatosis, hepatic injury, necroinflammation, and hepatic injury	Not evaluated
n-3 PUFA (2 g, 3 times daily) [44]	24 weeks	↑ PPARα	NAFLD patients with hyperlipidemia	↓ steatosis and hepatic injury	Not evaluated
Ω-3 FA (5 mL, thrice daily) [45]	24 weeks	↑ PPARα	NAFLD patients with dyslipidemia	↓ steatosis and hepatic injury	Not evaluated
Atorvastatin (20 mg/daily) [45]	24 weeks	↑ PPARα	NAFLD patients with dyslipidemia	↓ steatosis and hepatic injury	Not evaluated
Orlistat (120 mg, thrice daily) [45]	24 weeks	↑ PPARα	NAFLD patients with dyslipidemia	↓ steatosis and hepatic injury	Not evaluated

Table 1: Continued.

PPARα knockout					
Strategies	Time	Effect	Experimental model	Steatosis and hepatic injury	Regeneration
PPARα-knockout [23]	—	↓ PPARα	PPARα-null mice Warm ischemia (90 min)	↑ hepatic injury	Not evaluated
PPARα-knockout [46]	—	↓ PPARα	PPARα-null mice fed HF diet	↑ hepatic β-oxidation	Not evaluated
PPARα-knockout [47]	—	↓ PPARα	PPARα-null mice Partial hepatectomy	Not evaluated	↓ liver regeneration
PPARγ					
PPARγ activator					
Strategies	Time	Effect	Experimental model	Steatosis and hepatic injury	Regeneration
Rosiglitazone (10 mg/kg) [6]	30 min before ischemia	↑ PPARγ	PPARγ$^{\pm}$ mice	↓ hepatic injury	Not evaluated
Rosiglitazone (2.5 μmol/kg/d) [17]	1 week	↑ PPARγ	Ob/ob mice	↓ triglycerides	Not evaluated
Rosiglitazone (3 mg/kg/day) [48]	5 weeks	↑ PPARγ	PPARγ$^{fl/fl}$ mice fed HFD diet	↑ steatosis	Not evaluated
Rosiglitazone (1 mg/kg/day) [49]	12 weeks	↑ PPARγ	Obese C57BL/6J mice	↑ steatosis	Not evaluated
Rosiglitazone (10 mg/kg) [50]	2 days before surgery	↑ PPARγ	Mice partial hepatectomy	Not evaluated	↓ hepatic regeneration
Troglitazone (0.1%) + adPPARγ [51]	adPPARγ (5th day) troglitazone (5 days)	↑ PPARγ	PPARα-null mice fed CD diet	↑ steatosis	Not evaluated
Pioglitazone (500 μg/Kg) [52]	8 weeks	↑ PPARγ	Rat fed liquid diet + alcohol	↓ liver injury	Not evaluated
Pioglitazone (30 mg) [53]	96 weeks	↑ PPARγ	Patients with NASH	↓ steatosis	Not evaluated
Pioglitazone (25 mg/kg/day) [54]	5 days before surgery	↑ PPARγ	KK-A^{Y}, mice partial hepatectomy	Not evaluated	↑ hepatic regeneration
Pioglitazone (20 mg/kg) [7]	1.5 h before ischemia	↑ PPARγ	Mice Warm ischemia (60 min)	↓ hepatic injury	Not evaluated
Ang II blockers Captopril (100 mg/kg) or PD123319 (30 mg/kg) [36]	Immediately before ischemia	↑ PPARγ	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
EGF and IGF-1 (10 μg/L) [34]	24 h in UW solution	↑ PPARγ	Obese Zucker rats; isolated liver perfused (24 h cold ischemia)	↓ hepatic injury	Not evaluated
EGF (100 μg/Kg) [35]	3 doses (every 8 h) starting before surgery	↑ PPARγ	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
IGF-I (400 μg/Kg) [35]	2 doses (every 12 h) starting before surgery	↑ PPARγ	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
Adenovirus PPARγ + rosiglitazone (50 mg/kg/day) [55]	8 weeks	↑ PPARγ	C57BL/6J mice fed MCD diet	↓ steatohepatitis and fibrosis	Not evaluated
PC (5 min/10 min) [36]	Immediately before ischemia	↑ PPARγ	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated

Table 1: Continued.

PPARγ inhibitor					
Strategy	Time	Effect	Experimental model	Steatosis and hepatic injury	Regeneration
GW9662 (1 mg/kg) [14]	1 h before surgery	↓ PPARγ	Liver transplantation (6 h cold ischemia)	Does not change in hepatic injury	Not evaluated
GW9662 (1 mg/kg) [14]	1 h before surgery	↓ PPARγ	Steatotic liver transplantation (6 h cold ischemia)	↓ hepatic injury	Not evaluated
GW9662 (1 mg/kg, 3 times/week) [55]	8 weeks	↓ PPARγ	C57BL/6J mice fed MCD diet	↑ steatohepatitis, fibrosis and hepatic injury	Not evaluated
RBP4 (150 μg/kg) [14]	30 min before surgery	↓ PPARγ	Steatotic liver transplantation (6 h cold ischemia)	↓ hepatic injury	Not evaluated
PC (5 min/10 min) [14]	Immediately before ischemia	↓ PPARγ	Steatotic liver transplantation (6 h of cold ischemia)	↓ hepatic injury	Not evaluated
PPARγ inhibitor					
Strategies	Time	Effect	Experimental model	Steatosis and hepatic injury	Regeneration
PPARγ-knockout [56]	—	↓ PPARγ	Liver-specific PPARγ-null mice	↓ steatosis	Not evaluated

Tabla 2. Efecto de las estrategias que regulan los PPAR en el daño hepático, la esteatosis y la regeneración en modelos experimentales y en pacientes. Angiotensina II, Ang II; deficiente en colina, CD; factor de crecimiento epidérmico, EGF; dieta rica en grasas, HFD; factor de crecimiento similar a la insulina-1, IGF-1; deficiente en metionina y colina, MCD; esteatohepatitis no alcohólica, NASH; receptor activador de la proliferación de peroxisomas, PPARs; ácidos grasos poli-insaturados, PUFAs; precondicionamiento isquémico PC; proteína transportadora de retinol tipo 4: RBP4. Tabla obtenida del artículo: Elias-Miró M., y cols. 200d.

La interacción del PPAR-α con su sitio de reconocimiento de DNA esta notablemente mejorado por ligandos tales como los fármacos fibrato hipotrigliceridémicos, ácido linoleico conjugado y los PUFA. El descubrimiento del PPAR-α llevó rápidamente a la idea de que este era un factor de transcripción clave para inhibir genes que codifican para las proteínas de oxidación lipídica e inducir genes que codifican proteínas de oxidación lipídica. En línea con esta idea, estudios recientes sugieren que los ácidos grasos servirían como importantes mediadores de la expresión génica, trabajando vía los PPAR para controlar la expresión de los genes implicados en el metabolismo de los lípidos, la glucosa y la adipogénesis. Neschen y cols. (Neschen S., y cols. 2002) también demostraron que la administración de aceite de pescado aumenta los ácidos grasos hepáticos a través de su capacidad de funcionar como un ligando activador de PPAR-α, y de ese modo induce la transcripción de varios genes que codifican proteínas relacionadas con la oxidación de ácidos grasos. Otros estudios que examinan los efectos de la alimentación con aceite de pescado sobre la expresión de varios genes en ratones knockout para PPAR, indicaron claramente que la regulación genética mediada por este aceite implicaba por lo menos dos vías diferentes: la vía independiente de PPAR-α y la

dependiente de PPAR-α. Las enzimas de oxidación peroxisomasl (CYP4A2) y microsomal (AOX) són PPAR-α dependientes y sobrerreguladas por la dieta rica en aceite de pescado, mientras que las enzimas de síntesis lipídica (FAS y S14) están inhibidas y son independientes de PPAR-α. Esto indica que la regulación de la lipogénesis hepática *de novo* y la oxidación de ácidos grasos no son mediados a través de un factor común (por ejemplo, PPAR-α) (Elias-Miró M., y cols. 2012d).

Teniendo en cuenta todos estos. datos, la regulación de PPAR-α por PUFA, especialmente n-3 PUFA y posiblemente ácido linoleico conjugado, podría ofrecer una explicación de los beneficios reportados de estos ácidos grasos en diferentes patologías (Elias-Miró M., y cols. 2012d).

En pacientes obesos con NAFLD, el aumento de la producción de RLO conduce a un agotamiento de n-3 PUFA debido a una mayor peroxidación lipídica. Como el PPAR-α es activado mediante la unión directa a n-3 PUFA, su función hepática está comprometida en la obesidad. Esto impidió la sobre-regulación de genes implicados en el transporte de lípidos, la β-oxidación de ácidos grasos y la termogénesis, favoreciendo la síntesis de ácidos grasos y de triacilglicerol sobre la β-oxidación de ácidos grasos y promoviendo así la esteatosis hepática. Por lo tanto, la activación de PPAR-α mediante la suplementación con n-3 PUFA mejoró la esteatosis en pacientes obesos con NAFLD. En línea con esto, los pacientes con NASH tienen bajos niveles circulantes de n-3 PUFA, con el consiguiente aumento del ratio ácidos grasos n-6/n-3 y alteración del PPAR-α en el hígado de la actividad hepática de PPAR-α. Por otra parte, los PUFA activan al PPAR-α, aumentando así la β-oxidación de ácidos grasos, por lo que pueden cambiar el equilibrio de energía desde el almacenamiento hasta el consumo. Los n-3 PUFA reducen el daño hepático y los niveles de lípidos en suero de pacientes con NAFLD. En otro estudio, pacientes con hiperlipidemia, hipercolesterolemia y sobrepeso fueron tratados con ácidos grasos n-3, atorvastatina y orlistat, respectivamente. Los tres grupos de pacientes mostraron menos daño hepático, menor esteatosis y una reducción de los lípidos séricos (Elias-Miró M., y cols. 2012d).

Teniendo en cuenta que la esteatosis es un factor de riesgo en la cirugía del hígado, las estrategias encaminadas a reducir la esteatosis pueden aumentar la tolerancia de estos hígados a la I/R. Existen evidencias de que la regeneración del hígado se altera en ciertos modelos genéticos en que el hígado contiene exceso de grasa. Por ejemplo, los hígados esteatósicos de ratones Ob esteatósicos presentan una regeneración hepática defectuosa y alta mortalidad después de una hepatectomía parcial. Del mismo modo, se

observó un deterioro en la regeneración hepática en hígados esteatósicos sometidos a hepatectomía parcial con oclusión vascular en comparación con hígados no esteatósicos. Por el contrario, los fármacos que reducen la esteatosis hepática, tales como reguladores de PPAR-α, deben ser considerados con precaución en la cirugía hepática clínica, ya que como indican otros estudios, estrategias genéticas y farmacológicas que reducen la acumulación de lípidos también pueden dificultar la regeneración del hígado. Por lo tanto, una pregunta importante por resolver es hasta qué punto debemos reducir la esteatosis en los hígados esteatósicos para proteger este tipo de hígado. Otra cuestión interesante por resolver es si debemos reducir la esteatosis antes del procedimiento quirúrgico, y por lo tanto evitar la vulnerabilidad de los hígados esteatósicos al daño por I/R, o por el contrario, usar fármacos para reducir los triglicéridos hepáticos durante la cirugía, y así conservar la energía necesaria para la regeneración del hígado. Por otra parte, investigaciones encaminadas a evaluar si la administración a corto plazo de los agonistas de PPAR-α podrían aliviar la esteatosis hepática del hígado esteatósicos antes de la I/R serían de interés para la práctica clínica debido a que hay dificultados obvias acerca de la viabilidad de la administración de agonistas de PPAR-α a largo plazo en algunos procesos de I/R, en particular en trasplante hepático de donante cadavérico. Se trata de un procedimiento de emergencia en el que hay muy poco tiempo para pre-tratar el donante con agonistas de PPAR-α (Elias-Miró M., y cols. 2012d).

Varios estudios atribuyen al PPAR-γ un papel en el desarrollo de esteatosis por mecanismos que implican la activación de los genes lipogénicos y la lipogénesis *de novo*. En consonancia, la supresión selectiva de PPAR-γ en los hepatocitos protege a los ratones contra la esteatosis hepática inducida dietéticamente, lo que sugiere un papel pro-esteatósico del PPAR-γ. Del mismo modo, los ratones con PPAR-γ selectivamente silenciado en el hígado están protegidos frente a la esteatosis hepática. Además, el tratamiento de ratones ob/ob con rosiglitazona aumenta la esteatosis hepática. Por el contrario, existen diferentes resultados en relación al efecto del PPAR-γ en la esteatosis hepática. En efecto, ratones deficientes en PPAR-γ desarrollan un NAFLD más severo al ser tratados con una dieta MCD, mientras que una sobreexpresión de PPAR-γ mediada con adenovirus atenuaba la progresión del NASH. En línea con este hallazgo, el tratamiento con rosiglitazona previno el desarrollo de NASH en un modelo de ratones tratados con MCD, y se obtuvieron resultados similares utilizando pioglitazona, un agonista de PPAR-γ. Estos diferentes resultados pueden explicarse por diferencias en los estudios tales como, las especies usadas, el tipo de agonista de PPAR, el método

para inducir la esteatosis hepática, el tipo de estrategia genética utilizada para inducir la sobreexpresión de PPAR o deficiencia de este, así como las diferencias en los tiempos de pre-tratamiento de los fármacos utilizados (Elias-Miró M., y cols. 2012d).

1.8.4.- PPAR en la regeneración hepática

Estudios recientes han demostrado que la regeneración hepática está deteriorada en un número de modelos animales con enfermedad de hígado graso. Ratones knockout para PPAR-α sometidos a hepatectomía parcial tienen un deterioro de la capacidad de regenerar la masa hepática. Nuevos estudios indican que PPAR-α es un modulador crucial de la energía necesaria para la reparación de daños hepáticos. Por ejemplo, los hepatocitos en las regiones periportales, que se dividen y replican después de una hepatectomía parcial, requieren la oxidación mitocondrial de ácidos grasos para generar energía. El PPAR-α controla la expresión constitutiva de los genes implicados en la oxidación mitocondrial de ácidos grasos, incluyendo la carnitina palmitoiltransferasa-1. En ratones deficientes en PPAR-α, la regeneración hepática también se asocia con la expresión alterada de genes implicados en el control del ciclo celular y la señalización de citoquinas. Estudios con agonistas de PPAR-α indican que este regula genes implicados en el ciclo celular (Ccnd1 y cMyc), así como IL1r1 e IL-6R (**Figura 13**) (Elias-Miró M., y cols. 2012d).

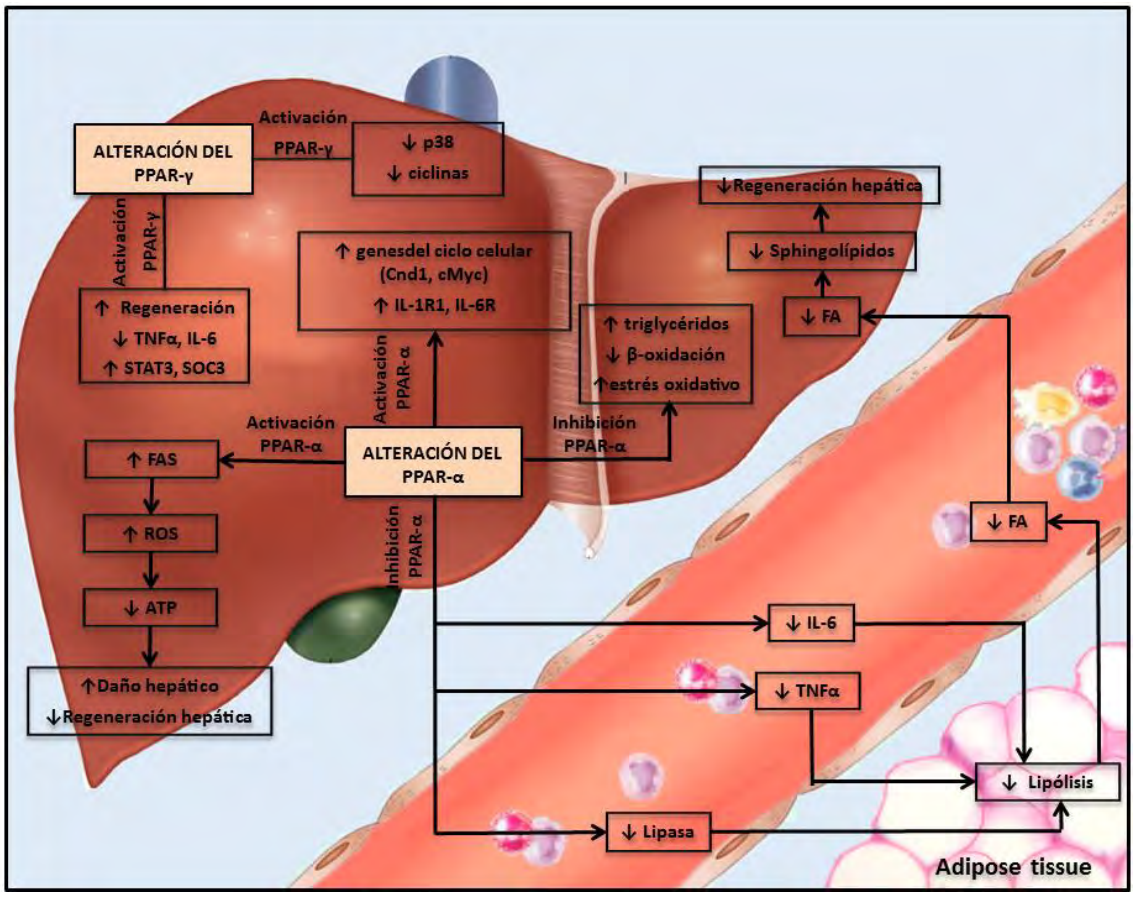


Figura 13. PPAR en la regeneración hepática.

Es bien conocido que el PPAR-α afecta a la transcripción de un número de genes implicados en el catabolismo lipídico y en la β-oxidación peroxisomal y mitocondrial, lo que resulta en la generación de ATP, que se requiere como "combustible" para la reparación y regeneración del hígado. Por el contrario, en condiciones en las que la función y/o expresión del PPAR-α se altera, tales como en esteatosis hepática o injertos de tamaño reducido, el metabolismo de los ácidos grasos se desvía hacia la acumulación de grasa inadecuadamente metabolizada, favoreciendo la generación de RLO. En consecuencia, se reduce la producción de ATP, y aumenta la necrosis hepatocitaria yse interrumpe la reparación del hígado (**Figura 13**) (Elias-Miró M., y cols. 2012d).

Los resultados anteriores indican que la regeneración hepática de los hígados esteatósicos es debido en parte a la inhibición del PPAR-α a través del eje AdipoR2. La inhibición del PPAR-α, aumentó la acumulación de triglicéridos en los hepatocitos e inhibió la expresión de las enzimas hepáticas que contribuyen a la oxidación de ácidos grasos (**Figura 13**). Esto se asoció con un aumento de la peroxidación de lípidos y una disminución de los niveles de antioxidantes (Elias-Miró M., y cols. 2012d).

En contraste con estos datos que indican los efectos beneficiosos del PPAR-α en la regeneración hepática, datos recientes indican que la activación de PPAR-α por benzafibrato tiene efectos negativos en la regeneración hepática, lo que puede atribuirse a la inhibición de la síntesis *de novo* de esfingolípidos. Presumiblemente, el benzafibrato afecta a la síntesis *de novo* de esfingolípidos mediante una disminució de ácidos grasos disponibles (**Figura 13**). La activación de PPAR-α por benzafibrato prácticamente previno el aumento post-operatorio de ácidos grasos plasmáticos no esterificados tras una hepatectomía parcial. Esto puede explicarse por la inhibición de la actividad de los ligandos del PPAR-α y sus propiedades anti-inflamatorias, lo cual disminuye la liberación de citoquinas tales como TNF e IL-6. Ambos eventos inhibieron la lipólisis en los adipocitos aislados, resultando en una menor liberación de ácidos grasos de fuentes extrahepáticas después de una hepatectomía parcial (Elias-Miró M., y cols. 2012d).

La actividad del PPAR-γ es probablemente regulada durante la regeneración hepática normal, y la interrupción de esta podría afectar la respuesta regenerativa. La pioglitazona mejoró el fallo regenerativo hepático en ratones obesos. Este efecto se asoció con una reducción de los niveles de TNF-α e IL-6. Sin embargo, se han obtenido resultados inconsistentes en relación con el efecto de PPAR-γ en la regeneración del hígado. En efecto, la rosiglitazona inhibe la proliferación de hepatocitos en ratones sometidos a hepatectomía parcial mediante la reducción de la expresión de p38 y de las ciclinas (Elias-Miró M., y cols. 2012d).

Teniendo en cuenta los resultados inconsistentes reportados hasta la fecha sobre el papel de los PPAR en la regeneración hepática, es difícil discernir si debemos inhibirlo o activarlo para promover la regeneración del hígado tras una cirugía hepática.

1.8.5.- Moduladores del PPAR en la práctica clínica

Basándose en los datos reportados en modelos experimentales (tal y como se ha descrito anteriormente), diferentes estrategias basadas en la regulación de los PPAR podrían ejercer efectos sobre la esteatosis, la inflamación, o la regeneración. Se desconoce si estos enfoques farmacológicos pueden derivar en tratamientos clínicos, sigue siendo un misterio. Por ejemplo, las tiazolidinedionas (TZD) no se deben aplicar en la cirugía hepática clínica debido a sus posibles efectos secundarios. Las TZD (pioglitazona, troglitazona, y rosiglitazona) son agonistas sintéticos del PPAR-γ que se utilizan ampliamente como agentes antidiabéticos. Sin embargo, el tratamiento prolongado de

ratones obesis y diabéticos con TZD resultó en el desarrollo de esteatosis severa, que puede conducir a esteatohepatitis y/o fibrosis. La administración de troglitazona se asoció con el desarrollo de la insuficiencia hepática aguda idiosincrásica y por lo tanto fue retirado del mercado. Se sabe que la pioglitazona y rosiglitazona de sus efectos hepatotóxicos (Elias-Miró M., y cols. 2012d).

Los agonistas del PPAR-α son clínicamente y funcionalmente relevantes como agentes terapéuticos contra la hiperlipidemia y agentes para reducir las complicaciones de la enfermedad vascular periférica en pacientes diabéticos. A pesar de su papel potencialmente beneficioso, los agonistas del PPAR-α deben utilizarse juiciosamente. La administración a corto plazo en humanos (1-10 días), sería poco probable que produjese efectos genotóxicos permanentes. Sin embargo, la exposición a largo plazo a estos fármacos, que sería necesaria para reducir la esteatosis hepática, puede resultar, entre otros efectos, en daño oxidativo del ADN (Elias-Miró M., y cols. 2012d).

Otros estudios también serán requeridos para esclarecer si los factores de crecimiento, los bloqueadores de Ang II o RBP4 pueden ser estrategias farmacológicas de protección más seguros para la regulación de los PPAR en la lesión hepática por I/R en la práctica clínica (**Figura 14**). Sin embargo, ninguna de las estrategias mencionadas anteriormente es específica para los PPAR (Elias-Miró M., y cols. 2012d).

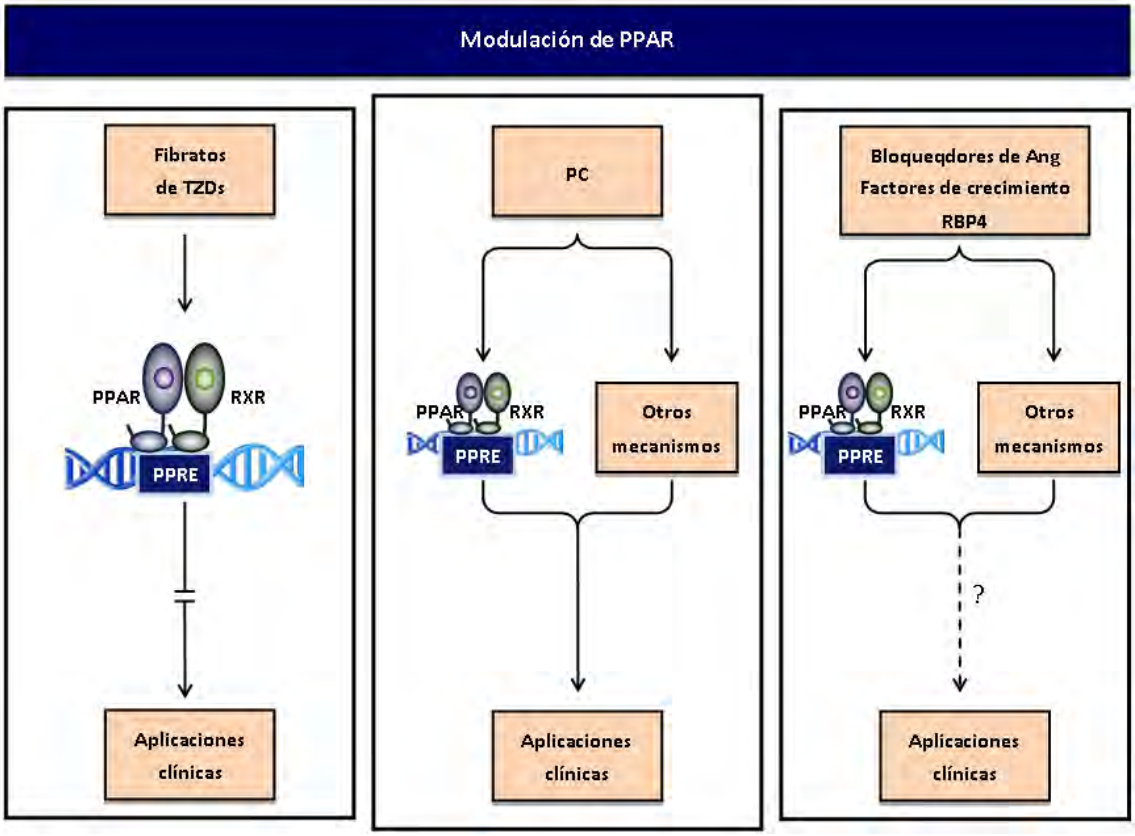


Figura 14. Aplicaciones clínicas de estrategias moduladoras de los PPAR.

2.- OBJETIVOS

La lesión por I/R hepática, inherente a la cirugía hepática, continúa siendo un problema sin resolver en la práctica clínica a pesar de los recientes avances en los tratamientos farmacológicos, quirúrgicos y del establecimiento de estrategias de terapia génica. Esta I/R hepática es especialmente problemática cuando se trata de hígados esteatósicos sometidos a resección parcial. La I/R afecta negativamente el proceso de la regeneración, siendo este efecto más evidente en presencia de esteatosis. En los últimos años, las investigaciones se han centrado en las adipocitoquinas como potenciales dianas terapéuticas en distintas patologías relacionadas con la obesidad y el síndrome metabólico. Desde hace tiempo se sabe que las adipocitoquinas regulan la esteatosis, la inflamación y la fibrosis, incluso han sido implicadas en la vulnerabilidad de los hígados esteatósicos a la lesión por I/R. El RBP4, adipocitoquina sintetizada en el hígado, ha demostrado ser efectivo un el modelo experimental de trasplante hepático, pero hasta la fecha no hay estudios sobre sus efectos en isquemia normotérmica asociada a las resecciones hepática. En base a estos antecedentes los objetivos de la presente tesis son los siguientes:

1. Investigar el papel del RBP4 en la lesión hepática y la regeneración en hígados esteatósicos y no esteatósicos sometidos a hepatectomía parcial con I/R.
2. Evaluar la relación existente entre el RBP4 y el retinol teniendo en cuenta que una de las principales funciones del RBP4 es transportar retinol en plasma.
3. Evaluar los posibles mecanismos de protección de estrategias farmacológicas que modulen el RBP4 y/o el retinol en la lesión y regeneración hepática en hígados esteatósicos y no esteatósicos sometidos a cirugía.

Los resultados que se obtengan a partir de tales investigaciones podrían derivar en nuevas terapias farmacológicas en las resecciones hepáticas clínicas, y en trasplante hepático con injertos de tamaño reducido. Esto incidiría en una mayor tolerancia de los injertos esteatósicos frente a la lesión por I/R y al fallo en la regeneración, y en una mayor supervivencia y menores complicaciones postquirúrgicas.

3.- MATERIALES Y MÉTODOS

3.1.- Animales de experimentación

Los animales utilizados en los diversos estudios experimentales de esta tesis fueron ratas macho de entre 14-16 semanas de edad de la cepa Zucker (Iffa-Credo, L'Abresle, France), mantenidas en el estabulario de la Facultad de Medicina de la Universidad de Barcelona, como mínimo durante una semana antes de llevar a cabo la intervención quirúrgica. Las condiciones ambientales se mantuvieron constantes: temperatura de 21-22ºC, humedad relativa del 70% y ciclos de luz-oscuridad de 12 h. Los animales se alimentaron ad libitum con una dieta con un 12% de grasa, un 28% de proteína y un 60% de hidratos de carbono (5001 rodent diet; PMI Inc., Brentwood, MO, USA). Todos los estudios se realizaron de acuerdo con las normas reguladoras de la Unión Europea para modelos de experimentación animal (Directiva 86/609/ECC).

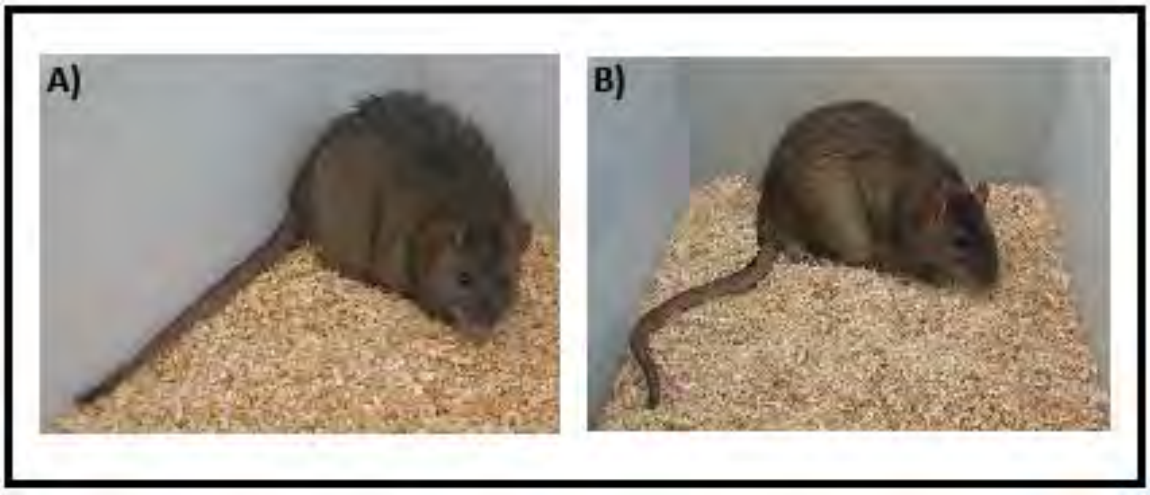


Figura 15. Animales de experimentación. A) Rata Zucker obesa (Ob) y B) Rata Zucker lean (Ln).

Las ratas de la cepa Zucker constituyen un modelo bien definido de obesidad inducida nutricionalmente. A diferencia de otros modelos experimentales de esteatosis que utilizan una dieta deficiente en colina-metionina o rica en alcohol; la esteatosis hepática que se produce en las ratas Zucker no está asociada con inflamación hepática (Selzner M., y cols. 2000; Koteish A., y cols. 2001). Las ratas Zucker presentan dos fenotipos diferentes: las ratas Zucker heterocigotos (fa/-) de fenotipo delgado (Ln) (**Figura 15**), que tienen receptores cerebrales de leptina y mantienen un fenotipo delgado toda su vida; y las ratas Zucker homocigotos (fa/fa) de fenotipo obeso (Ob), que carecen de receptores cerebrales de leptina y desarrollan obesidad desde las 8 semanas de edad debido al incremento en la ingesta de comida y a la disminución del gasto calórico.

La diferencias de esteatosis entre los animales Ln y los Ob se determinó utilizando una tinción específica para lípidos, RedOil. Los animales Ln no muestran evidencia de esteatosis. Por lo contrario, en las ratas Ob se observó una infiltración grasa severa (entre el 60%-70% de esteatosis) macrovesicular y microvesicular en el citoplasma de los hepatocitos (**Figura 16**).

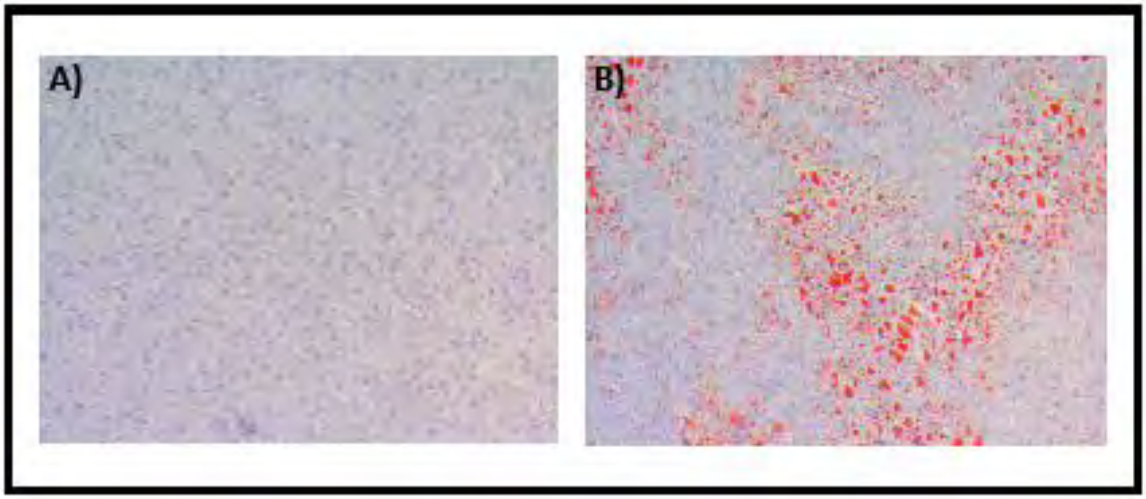


Figura 16. Diferencias histológicas de esteatosis entre ratas Zucker Ob y ratas Zucker Ln mediante tinción RedOil. A) Rata Zucker Ln sin evidencias de esteatosis y B) Rata Zucker Ob con infiltración grasas en los hepatocitos.

3.2.- Modelo experimental

3.2.1.- Anestesia

Todos los procedimientos se llevaron a cabo bajo anestesia inhalatoria. La inducción anestésica se realizó con isofluorano (Forane®, Abbot laboratorios, Chicago, IL., USA) al 4% con un flujo de oxígeno de 2.5 a 3L/min.

3.2.2.- Procedimiento quirúrgico de la hepatectomía parcial con isquemia y reperfusión

Una vez anestesiada la rata, se rasuró el abdomen y se colocó de cúbito supino sobre la mesa de operaciones. El campo quirúrgico se lavó con povidona iodada. Se realizó una laparotomía vertical, siguiendo la línea blanca que se aprecia a lo largo del vientre del animal. A continuación se seccionó los ligamentos hepáticos. Posteriormente se clampó el lóbulo triangular con las pinzas Statinsky, y la vena porta con un clamp tipo "Bulldog". Cuando empezó el tiempo de isquemia se anudó y seccionó los lóbulos medio y el triangular. Cuando se cumple la hora de isquemia, se retiró el clamb vascular. Rápidamente anudó y seccionó el lóbulo inferior derecho y los lóbulos caudatos. Una vez cerrado el animal, se empezó a contabilizar las horas de reperfusión para recoger las muestras (**Figura 17**).

Figura 17. Hepatectomía parcial con isquemia y reperfusión. A) Lóbulos hepáticos; B) Lóbulo derecho-triangular clampado; C) Rata al finalizar la hepatectomía parcial con I/R.

3.3.- Diseño experimental

En la **Tabla 3** se presenta un resumen del diseño experimental y las determinaciones analíticas realizadas en este estudio. Los protocolos experimentales se describen en detalle a continuación.

Protocolo 1: Efectos del RBP4 en hepatectomía parcial con I/R

Para evaluar el papel del RBP4 en la lesión hepática y la regeneración en condiciones de hepatectomía parcial con I/R, se realizaron los siguientes grupos experimentales:

1. Sham (n=12, 6 Ln y 6 Ob): Los animales Ln y Ob se sometieron a anestesia y laparotomía transversal.
2. Hepatectomía parcial con I/R (PH+I/R) (n=12, 6 Ln y 6 Ob): Los animales Ln y Ob se sometieron a una resección hepática parcial (del 70 %) con 60 minutos de isquemia y posterior reperfusión de 24 horas.
3. Hepatectomía parcial con I/R + RBP4 (PH+I/R+RBP4) (n=12, 6 Ln y 6 Ob, por cada dosis evaluada): Igual que el grupo 2, pero tratado con diferentes dosis de RBP4 (AdipoGen Inc., Seoul, Korea). Dosis de RBP4: 5, 10, 25, 50 y 150µg/kg.

Protocolo 2: Efectos del retinol en hepatectomía parcial con I/R

Para evaluar el papel del retinol en la lesión hepática y la regeneración en condiciones de hepatectomía parcial con I/R, se realizaron los siguientes grupos experimentales:

4. Hepatectomía parcial con I/R + retinol (PH+I/R+Retinol) (n=12, 6 Ln y 6 Ob, por cada dosis evaluada): Igual que el grupo 2, pero tratado con diferentes dosis de retinol (Sigma-Aldrich, España). Dosis de retinol: 5, 10, 15 y 30mg/kg.

Protocolo 3: Efectos del RBP4 y retinol en combinación en hepatectomía parcial con I/R

Para evaluar el efecto de la combinación de RBP4 y retinol en combinación en la lesión hepática y la regeneración en condiciones de hepatectomía parcial con I/R, se realizaron los siguientes grupos experimentales.

5. Hepatectomía parcial con I/R + RBP4 (PH+I/R+RBP4) (n=12, 6 Ln y 6 Ob): Igual que el grupo 2, pero con administración previa de RBP4 (AdipoGen Inc.,Seoul, Korea), a dosis de 5 μg/kg por vía intravenosa.

6. Hepatectomía parcial con I/R + retinol (PH+I/R+Retinol) (n=12, 6 Ln y 6 Ob): Igual que el grupo 2, pero con administración previa de retinol (Sigma-Aldrich, España), a dosis de 10 mg/kg por vía intraperitoneal.

7. Hepatectomía parcial con I/R + RBP4 + retinol (PH+I/R+RBP4+Retinol) (n=12, 6 Ln y 6 Ob): Igual que el grupo 2, pero con administración previa de RBP4 (AdipoGen Inc., Seoul, Korea), a dosis de 5 μg/kg por vía intravenosa y retinol (Sigma-Aldrich, España), a dosis de 10 mg/kg por vía intraperitoneal.

Protocolo 4: Efecto del retinol sobre el daño hepático, la actividad proliferativa y el grado de esteatosis, en función del tiempo de reperfusión

Para evaluar el papel del retinol en función del tiempo de reperfusión en la lesión hepática, la regeneración y el grado de esteatosis en condiciones de hepatectomía parcial con I/R, se realizaron los siguientes grupos experimentales.

8. Hepatectomía parcial con I/R en función del tiempo de reperfusión ($PH+I/R_{(RT)}$) (n=12, 6 Ln y 6 Ob, por cada tiempo de reperfusión evaluado): Igual que el grupo 2, pero con administración de Bromodeoxirudine (BrdU) a dosis de 50 mg/kg 1 hora antes de la cirugía intraperitoneal.

9. Hepatectomía parcial con I/R + retinol en función del tiempo de reperfusión ($PH+I/R+Retinol_{(RT)}$) (n=12, 6 Ln y 6 Ob, por cada tiempo de reperfusión evaluado): Igual que el grupo 4, pero con administración de Bromodeoxirudine (BrdU) a dosis de 50 mg/kg 1 hora antes de la cirugía intraperitoneal.

Protocolo 5: Mecanismo de acción del retinol en el hígado esteatósico en hepatectomía parcial con I/R

Para evaluar los mecanismos de acción del retinol en la lesión hepática y la regeneración en condiciones de hepatectomía parcial con I/R, se realizaron los siguientes grupos experimentales.

10. Hepatectomía parcial con I/R + retinol + antagonista de retinol (Retinol+antagPPAR-γ) (n=6 Ob): Igual que el grupo 2, pero con administración

previa de retinol (Sigma-Aldrich, España), a dosis de 10 mg/kg por vía intraperitoneal y el antagonista de PPAR-γ, GW9662 (Alexis Biochemicals, Lausen, Switzerland), a dosis de 1 mg/kg vía intraperitoneal.

Diseño experimental	
Protocolo 1. Efectos del RBP4 en PH+ I/R	
1. Sham (n=12), dividido en dos grupos; 1.1 Sham (n=6 Ln) 1.2 Sham (n=6 Ob) 2. PH+I/R (n=12), dividido en dos grupos; 2.1 PH+I/R (n=6 Ln) 2.2 PH+I/R (n=6 Ob) 3. RBP4, dividido en dos grupos; 3.1 RBP4 (n=6 Ln) 3.2 RBP4 (n=6 Ob)	 Laparotomía transversal de animales Ln. Laparotomía transversal de animales Ob. Animales Ln sujetos a hepatectomía parcial con isquemia y reperfusión. Animales Ob sujetos a hepatectomía parcial con isquemia y reperfusión. Como en el grupo 2.1, pero tratados con distintas dosis de RBP4 (5, 10, 25, 50 y 150µg/kg). Como en el grupo 2.2, pero tratados con distintas dosis de RBP4 RBP4 (5, 10, 25, 50 y 150µg/kg).
Determinaciones: Grupos 1 y 2: Grupos 1, 2 y 3:	 RBP4 en hígado y plasma Daño hepático (transaminasas plasmáticas y grado de lesión celular), regeneración hepática (HGF, TGF-β y porcentaje de hepatocitos Ki-67 positivos) y supervivencia (durante 14 días tras la cirugía).
Protocolo 2. Efectos del retinol en PH+ I/R	
4. Retinol (n=12), dividido en dos grupos; 4.1 Retinol (n=6 Ln) 4.2 Retinol (n=6 Ob)	 Como en el grupo 2.1, pero tratados con distintas dosis de retinol (5, 10, 15 y 30mg/kg). Como en el grupo 2.2, pero tratados con distintas dosis de retinol (5, 10, 15 y 30mg/kg).
Determinaciones: Grupos 1 y 2: Grupos 1, 2 y 4:	 Retinol y ésteres de retinilo. Daño hepático (transaminasas plasmáticas y grado de lesión celular), regeneración hepática (HGF, TGF-β y porcentaje de hepatocitos Ki-67 positivos) y supervivencia (durante 14 días tras la cirugía).
Protocolo 3. Efectos la combinación de RBP4 y retinol en PH+ I/R	
5. RBP4 (n=12, 6 Ln y 6 Ob) 6. Retinol (n=12, 6 Ln y 6 Ob) 7. RBP4+Retinol (n=12, 6 Ln y 6 Ob)	Como en el grupo 2, pero tratados con RBP4 (5 µg/kg). Como en el grupo 2, pero tratados con retinol (10 mg/kg). Como en el grupo 2, pero tratados con RBP4 (5 µg/kg) y retinol (10 mg/kg).
Determinaciones: Grupos 1, 2.2, 5, 6 y 7:	 Daño hepático (transaminasas plasmáticas y grado de lesión celular), regeneración hepática (HGF, TGF-β y porcentaje de hepatocitos Ki-67 positivos), supervivencia (durante 14 días tras la cirugía), retinol y ésteres de retinilo en hígado y plasma, RBP4, TTR y relación RBP4/retinol en plasma.
Protocolo 4. Efecto, dependiente del tiempo de reperfusión, del retinol	
8. PH+I/$R_{(TR)}$ (n=36, 18 Ln y 18 Ob) 9. $Retinol_{(TR)}$ (n=36, 18 Ln y 18 Ob)	Como en el grupo 2, pero con tiempos de reperfusión de 12, 24 y 48 horas. Como en el grupo 4, pero con tiempos de reperfusión de 12, 24 y 48 horas.
Determinaciones: Grupos 8 y 9:	 Daño hepático (transaminasas plasmáticas), regeneración hepática (índice mitótico y porcentaje de hepatocitos BrdU positivos), triglicéridos en hígado y plasma, y esteatosis hepática (RedOil).
Protocolo 5. Mecanismo de acción del retinol en el hígado esteatósico en PH+ I/R	
10. Retinol+antagonistsPPAR-γ (n= 6 Ob)	Como en el grupo 2.2, pero tratados con retinol (10 mg/kg) y antagonista de PPAR-γ (1 mg/kg).
Determinaciones: Grupos 1.2, 2.2 y 6: Grupos 1.2, 2.2, 6 y 10:	 Estrés oxidativo (MDA y nitrotirosinas), PPAR-α y PPAR-γ. Daño hepático (transaminasas plasmáticas y grado de lesión celular), regeneración hepática (HGF, TGF-β y porcentaje de hepatocitos Ki-67 positivos), triglicéridos en hígado y plasma, y esteatosis hepática (RedOil).

Tabla 3. Diseño experimental y determinaciones analíticas

3.4.- Recogida y procesamiento de las muestras

En todos los grupos experimentales descritos, se recogieron muestras de plasma e hígado después de 24 horas de reperfusión. En el grupo Sham, las muestras de hígado y tejido se tomaron 24 horas después de la laparotomía. En el protocolo 4, las muestras fueron obtenidas a las 12, 24 y 48 horas de reperfusión. Las muestras de sangre se tomaron de la vena cava inferior infra-hepática, utilizando heparina como anticoagulante. Estas muestras se mantuvieron a 4ºC hasta que se centrifugaron a 3000 rpm durante 10 minutos a 4ºC para obtener el plasma. El plasma se mantuvo congelado a -80ºC hasta la realización de las correspondientes determinaciones analíticas. Inmediatamente después de recoger muestras de tejido hepático, una parte de este se congeló con nieve carbónica y se mantuvo a -80ºC hasta su posterior procesamiento. Otra parte del tejido hepático se fijó en formaldehído tamponado al 4% para realizar después el estudio histológico e inmunohistoquímico.

Para evaluar la lesión hepática se realizaron las siguientes determinaciones: transaminasas y estudio histológico del tejido hepático en cortes histológicos de hígado. Para evaluar la regeneración hepática se realizaron las siguientes determinaciones: porcentaje de hepatocitos positivos por Ki-67, porcentaje de hepatocitos positivos por BrdU, índice mitótico, y los niveles de HGF y TGF-β. Para evaluar la expresión y función del RBP4 y del retinol se determinaron los niveles de RBP4, retinol, ratio RBP4/retinol, ésteres de retinilo y TTR en muestras de tejido hepático. Para evaluar el grado de esteatosis se realizaron las siguientes determinaciones: Triglicéridos y RedOil.

La dosis y tiempo de pre-tratamiento utilizado para el antagonista de PPAR-γ (GW9662) fueron seleccionados en base a estudios anteriores (Casillas-Ramírez A., y cols. 2011). Los grupos control se realizaron con el vehículo correspondiente de cada fármaco (salino para RBP4 y dimetil sulfóxido para retinol y el antagonista de PPAR-γ).

3.5.- Determinaciones analíticas

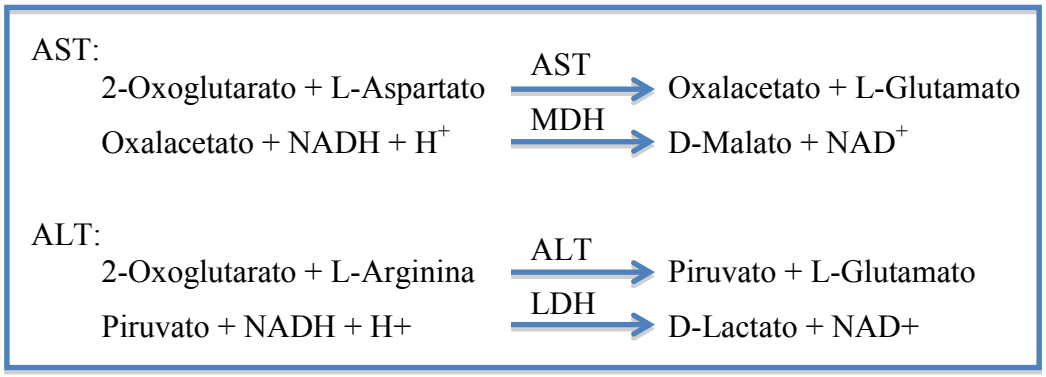
3.5.1.- Parámetros de lesión hepática

Para evaluar la lesión hepática se realizaron las determinaciones que se describen a continuación.

3.5.1.1.- Actividad de las transaminasas

Las transaminasas aspartato amino transferasa (AST) y alanina aminotransferasa (ALT) son enzimas que se encargan de realizar la conversión de un aminoácido en un ácido carboxílico, participan en el metabolismo de los aminoácidos y son muy abundantes en hígado. En condiciones normales estas enzimas se encuentran en el citoplasma, pero al existir una lesión hepática son liberadas al plasma. De esta forma la determinación de los niveles de estas enzimas se ha utilizado como parámetro de la lesión hepática.

La actividad de estas enzimas se determinó mediante kit comercial (RAL, Barcelona, España). El ensayo enzimático mide la desaparición de Nicotinamida adenina dinucleótido en su forma reducida (NADH) mediante espectrofotometría a una longitud de onda de 365 nm, según las siguientes reacciones:



NAD+: Nicotinamida adenina dinucleótido en su forma oxidada.

Los resultados obtenidos se expresaron en UI/L.

3.5.1.2.- Estudio histológico de la lesión hepática

Grado de lesión: Las muestras se procesaron según procedimientos estándar para su estudio mediante microscopía óptica. Inmediatamente después de la extracción las muestras se fijaron al menos durante 24 h en formaldehído tamponado al 4% (Panreac, Barcelona, España). Tras la inclusión en parafina, las muestras se cortaron con un

microtomo en secciones de 3-4 μm. Se realizó la tinción de hematoxilina y eosina (HyE) siguiendo los procedimientos convencionales.

Para evaluar la severidad de la lesión hepática, los cortes se clasificaron por un método de contaje utilizando la siguiente escala: grado 0, lesión mínima o sin evidencia de lesión:

1. Grado 1, lesión consistente en vacuolización citoplasmática y picnosis nuclear focal;
2. Grado 2, lesión de moderada a severa con picnosis nuclear extensa, hipereosinofilia citoplasmática y pérdida del contorno celular;
3. Grado 3, necrosis severa con desaparición de los cordones de hepatocitos, hemorragias e infiltrados de neutrófilos.

3.5.2.- Parámetros de regeneración hepática

Para evaluar los niveles de regeneración hepática se realizaron las determinaciones que se describen a continuación.

3.5.2.1.- Ensayo inmunoenzimático de HGF

Se homogenizaron las muestras hepáticas en un tampón que contenia Tris hidroxido 20 mM, cloruro de sodio 2M, Tween 80 al 0.1%, EDTC 1mM y PMSF 1mM a un pH de 7.5 con la ayuda de un homogenizador polytron. Seguidamente, se centrifugaron los homogenados a 20000g durante 60 minutos a 4ºC. Se recogió el sobrenadante y se congeló a -80ºC.

Para determinar los niveles de HGF en tejido se utilizó un kit comercial del Institute of Immunology (Tokyo, Japón).

3.5.2.2.- Ensayo inmunoenzimático de TGF-β

Para la determinación del TGF-β, las muestras hepáticas se homogenizaron en un tampón fosfato que contenia PMSF 2mM y pepstatina A 1mg/ml a 4ºC con la ayuda de un homogenizador Polytron. Seguidamente, las muestras se centrifugaron a 10000g durante 10 minutos a 4ºC y se recogió el sobrenadante del cual se determinó el TGF-β activo. Para medir el TGF-β total (activo y latente), las muestras se acidificaron con hidróxido clorhídrico 1M y después se neutralizó con hidróxido sódico 1M.

Para la determinación de los niveles totales y los activos de TGF-β se utilizó un kit de la casa comercial R&D Systems (Francia).

3.5.2.3.- Estudio histológico de la regeneración hepática

Porcentaje de hepatocitos Ki-67 positivos: La expresión de la proteína Ki-67 está estrictamente asociada con la proliferación celular. El hecho de que la proteína Ki-67 esté presente durante todas las fases activas del ciclo celular (G1, S, G2, y la mitosis), pero esté ausente de las células en reposo (G0), hace que sea un excelente marcador para la determinar la proporción de células que se están dividiendo.

Las muestras de tejido hepático se fijó en formaldehído tamponado al 4% (Panreac, Barcelona, España) y pasadas 24 horas se incluyeron en parafina. Las muestras se cortaron con un microtomo en secciones de 3-4 μm de grosor. Los cortes se desparafinaron con xilol y se hidrataron con soluciones de etanol de concentraciones decrecientes.

La inmunohistoquímica se llevó a cabo mediante un kit comercial (DAKO Envision+System, peroxidase (DAB); Dako, Alemania) siguiendo las instrucciones del fabricante. En primer lugar se bloqueó la peroxidada interna de la muestra. Se incubó con el anticuerpo primario anti Ki-67 (clon SP6; Abcam, Cambrige, MA). Después de incubar con el anticuerpo secundario, las muestras se trataron con DAB y el sustrato cromógeno, para dar un color marrón a las células en división. Se tiñeron los cortes con hematoxilina para dar una tinción de contraste y se montaron los portaobjetos.

El índice de Ki-67 se determinó mediante el conteo de núcleos teñidos en 30 campos de gran aumento. Y los datos se expresaron como el porcentaje de células teñidas respecto al número total de hepatocitos.

Porcentaje de hepatocitos BrdU positivos: El 5-bromo-2'-desoxiuridina (BrdU) es un nucleósido sintético análogo de la timidina. Durante la replicación del ADN puede ser incorporado en sustitución de la timidina. Por lo tanto, el BrdU también es un excelente marcador para la determinación de la proliferación celular. Una hora antes del sacrificio del animal, se inyectó BrdU (Sigma-Aldrich, España) en PBS por vía intraperitoneal (50 mg/kg). Las muestras se procesaron según procedimientos estándar para su estudio mediante microscopía óptica. Inmediatamente después de la extracción las muestras se fijaron durante 24 horas en formaldehído tamponado al 4% (Panreac, Barcelona, España). Tras la inclusión en parafina, las muestras se cortaron con un microtomo en

secciones de 3-4 μm de grosor. Los cortes se desparafinaron con xilol y se hidrataron con soluciones de etanol de concentraciones decrecientes.

La inmunohistoquímica se llevó a cabo mediante un kit comercial (DAKO Envision+System, peroxidase (DAB; Dako, Alemania) siguiendo las instrucciones del fabricante. En primer lugar se bloqueó la peroxidada interna de la muestra. Se incubó con el anticuerpo primario anti BrdU (clon Bu20a; DAKO, Alemania). Después de incubar con el anticuerpo secundario, las muestras se trataron con DAB y el sustrato cromógeno que da color marrón a las células en división. Se tiñeron los cortes con hematoxilina para dar una tinción de contraste y se montaron los portaobjetos.

El índice de BrdU se determinó mediante el contaje de núcleos teñidos en 30 campos de gran aumento. Y los datos se expresaron como el porcentaje de células teñidas respecto al número total de hepatocitos.

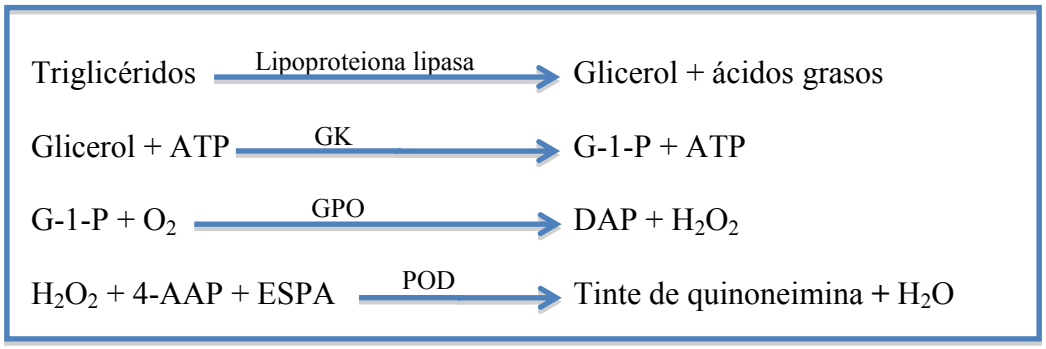
3.5.3.- Parámetros para determinar el grado de esteatosis

Para evaluar los niveles de esteatosis hepática se realizaron las determinaciones que se describen a continuación.

3.5.3.1.- Triglicéridos

Para llevar a cabo la extracción de lípidos totales en tejido hepático, las muestras de hígado se pesaron y se homogenizaron en una mezcla cloroformo: metanol en una relación 2:1 respectivamente. Una vez homogenizados se añadió metanol y posteriormente se procedió a centrifugar a 3000 g durante 15 minutos. Se descartó el precipitado. Posteriormente se añadió agua en proporción 1/5 del volumen del homogenizado y se procedió a centrifugar a 3000 g durante 15 minutos para separar las fases. La fase superior se descartó ya que es la fase no lipídica, y la fase inferior es la fase lipídica que es la que necesitamos. Para acabar de extraer, se hicieron tres lavados de la interfase con una mezcla cloroformo: metanol: agua que contiene la relación siguiente de los tres solventes 3:48:47 respectivamente, y en cada lavado se descarto la fase superior. Finalmente se añadió metanol y este extracto se hizo servir para determinar el contenido en triglicéridos del hígado (Folch J y cols 1957).

La determinación de los triglicéridos se llevó a cabo usando un kit comercial (Sigma, España) que se basa en las siguientes reacciones enzimáticas:



G-1-P: glicerol-1-fosfato
GK: glicerol kinasa
GPO: glicerol fosfato oxidasa
DAP: dihidroxiacetona fosfato
POD: peroxidasa
4-AAP: 4-aminoantipirina
ESPA: N-etil-N-(3-sulfopropil) m-anisidina de sodio

Se lee la absorbancia de la quinoneimina a una longitud de onda de 540 nm. El incremento de absorbancia es directamente proporcional a la concentración de triglicéridos en la muestra.

3.5.3.2.- Estudio histológico de la esteatosis hepática

RedOil: El RedOil es un colorante liposoluble que se utiliza para la tinción de triglicéridos y lípidos; por esto es una buena tinción para estudiar la grasa infiltrada en el tejido hepático. Las muestras se procesaron según procedimientos estándar para su estudio mediante microscopía óptica. Inmediatamente después de la extracción las muestras se fijaron al menos durante 24 h en formaldehído tamponado al 4% (Panreac, Barcelona, España). Tras la inclusión en parafina, las muestras se cortaron con un microtomo en secciones de 3-4 μm. Se realizó la tinción de RedOil siguiendo los procedimientos convencionales.

El índice de esteatosis hepática se determinó mediante el conteo de células con gotas lipídicas teñidas en 30 campos de gran aumento. Y los datos se expresaron como el porcentaje de células teñidas respecto al número total de hepatocitos.

3.5.4.- Parámetros para determinar los niveles de RBP4

Para evaluar los niveles de RBP4 presentes en el tejido hepático, se realizaron las determinaciones que se describen a continuación.

3.5.4.1.- Ensayo inmunoenzimático de RBP4

Para determinar los niveles de RBP4, las muestras de tejido hepático se homogenizaron en frío en tampón RIPA (Tris-HCl 50 mM, NaCl 150 mM, SDS 0.1%, Deoxicolato de sodio 1% y Nonidet P40 1%) conteniendo también EDTA 5mM, Na3VO4 1 mM, NaF 50 mM, DTT 1 mM y una tableta del cóctel de inhibidores de proteasas Complete por cada 50 ml de tampón (Roche, Basilea, Suiza). Los homogenizados se incubaron 20 minutos en hielo y después se centrifugaron a 10 000 g durante 30 min a 4ºC. Se recuperaron los sobrenadantes y se determinó el RBP4 en los mismos utilizando un kit comercial (AdipoGen, Seúl, Corea) y siguiendo las instrucciones del fabricante.

3.5.4.2.- RT-PCR de RBP4

La detección de la expresión génica (mRNA) de RBP4 se determinó en tejido hepático utilizando la técnica RT-PCR. Se realizó la extracción de RNA de tejido hepático utilizando los kits RNeasy Lipid Tissue kit para hígados esteatósicos y el RNeasy Mini kit para los hígados no esteatósicos (Quiagen, Hilden, Alemania). Después se realizó la cuantificación del RNA obtenido y se verificó también la calidad del RNA. A continuación se realizó la retrotranscripción del RNA utilizando el kit comercial Ready-To-go You-Prime First Strand Beads (GE Healthcare Bio-Sciences, Little Chalfont, Reino Unido). Finalmente se realizó RT- PCR a partir del cDNA obtenido y usando sondas/primer TaqMan Gene Expression Assay prediseñadas y validadas por la casa comercial que las sintetiza (Applied Biosystems, Foster City, CA, USA). Las sondas/primer utilizadas en esta determinación fueron las siguientes: Rn01451317_g1 para RBP4 y Rn00667869_m1 para β-Actina. También se llevaron a cabo experimentos control de la eficiencia del ensayo. La cuantificación de la expresión génica de RBP4 se realizó aplicando el método de ΔΔCt usando la β-Actina como gen de referencia o normalizador, y el grupo experimental Sham (grupo experimental 1) como calibrador para realizar el cálculo comparativo. La Real Time PCR se llevó a cabo en un termociclador iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Los resultados se expresaron en función del número de incrementos de la expresión del mRNA (con respecto al grupo calibrador).

3.5.5.- Parámetros para determinar los niveles de retinol y los ésteres de retinilo

El retinol y los ésteres de retinilo se midieron en hígado y plasma con una cromatografía de fase inversa de alto rendimiento (Waters, Alemania). Se realizó una extracción orgánica de la vitamina A, tanto plasmática como hepática, y se separó en una columna C18 (Repro-Sil 70, Alltech Grom, Rottenburg-Hailfingen, Alemania). El retinol y los ésteres de retinilo se identificaron por la comparación de su tiempo de retención con estándares externos [retinol y palmatato de retinil (Sigma, Deisenhofen, Germany); retinil oleato y estearato de retinil (Hoffmann-La Roche, Basel, Switzerland)] mediante el uso de un detector de fotodiodos (modelos PDA 996, Walters, Alemania). El retinol y los ésteres de retinilo se cuantificaron por la medición de la absorción a 325 nm. La exactitud y precisión se verificó mediante un material de referencia estándar (SMR 968a liposoluble vitaminas en el suero humano; Instituto Nacional de Estándares y Tecnología (NIST), Gaithersburg, Estados Unidos de América). La tasa de recuperación fue mayor del 95%, y el coeficiente de variabilidad entre las carreras fue del 4%.

3.5.6.- Parámetros para determinar los niveles de PPAR-α y PPAR-γ

La determinación de PPAR-α y PPAR-γ se realizó en tejido hepático, para lo cual las muestras se homogenizaron a 4º C en un tampón RIPA que contenía Tris-HCl 50 mM, NaCl 150 mM, SDS 0.1%, Deoxicolato de sodio 1% y Nonidet P40 1%, EDTA 5mM, Na3VO4 1 mM, NaF 50 mM, DTT 1 mM y una tableta del cóctel de inhibidores de proteasas Complete por cada 50 ml de tampón (Roche, Basilea, Suiza). Los homogenizados se centrifugaron a 12 000 g durante 10 min a 4º C, se separaron los sobrenadantes y se determinó la concentración de proteínas totales en los mismos tal y como se describe en el apartado correspondiente.

A continuación se procedió a realizar la técnica de western blot para la detección de PPAR-α y PPAR-γ. Con tal fin se mezclaron alícuotas de los homogenizados con un tampón de carga de electroforesis que contenía β-mercaptoetanol y tampón Laemli en proporción 1:20. La proporción del homogenizado y el tampón de carga fue 1:1. Las proteínas se desnaturalizaron calentando la mezcla a 95ºC durante 10 min. Se cargaron 100 μg de proteína y se separaron las proteínas de acuerdo a su peso molecular por electroforesis en un gel de poliacrilamida/SDS al 10% a un voltaje constante de 56 mV

en el Upper gel, y de 105 mV cuando las muestras se encontraban en el Lower gel. Posteriormente las proteínas fueron transferidas a una membrana de polifluoruro de vinilideno (PVDF) con una intensidad de 120 mA durante hora y media. Después de esto, los geles se tiñeron con comassie para asegurar que se había cargado igual cantidad de proteína y que las proteínas se habían transferido a la membrana de PVDF. Las membranas se incubaron en un tampón de bloqueo formado por una solución salina tamponada con Tris que contiene Tween-20 (TTBS) a pH=7.5 y leche en polvo no grasa al 3% durante 60 min a temperatura ambiente. A continuación las membranas se incubaron durante toda la noche a 4ºC con el anticuerpo primario policlonal anti-PPAR-α (Abcam, Cambrige, MA) o el anticuerpo primario policlonal anti-PPAR-γ (Abcam, Cambrige, MA) preparados en una dilución 1:200 y 1:125, respectivamente, disueltos en tampón de bloqueo. Al día siguiente, las membranas se lavaron con TTBS pH=7.5 y después se incubaron con el anticuerpo secundario durante 60 minutos a temperatura ambiente. En ambos casos se utilizó un anticuerpo secundario anti-IgG de conejo conjugado con peroxidasa (Bio-Rad Laboratories, Hercules, CA, USA) que fue preparado en dilución 1:2000 disuelto en tampón de bloqueo. Después de lavar la membrana con TTBS y finalmente con solución salina tamponada con Tris (TBS), se procedió a realizar la detección de las proteínas mediante el kit de quimioluminiscencia Immun-Star HRP (Bio-Rad Laboratories, Hercules, CA, USA) siguiendo las instrucciones del fabricante. Como marcador de peso molecular se utilizó el estándar preteñido Kaleidoscope (Bio-Rad Laboratories, Hercules, CA, USA) y se utilizó un control positivo para PPAR-α (Abcam, Cambrige, MA) y otro para PPAR-γ (Abcam, Cambrige, MA) que se analizó en paralelo a las muestras. Para PPAR-α se detectó una banda a 52 kDa, y para PPAR-γ a 54 kDa.

La estimación cuantitativa de las proteínas en las membranas se estandarizó respecto a la β-actina. Para ello las membranas se re-incubaron con un anticuerpo primario anti-β-actina de rata (Sigma-Aldrich, España) preparado en dilución 1:1000 disuelto en tampón que contenía TTBS pH=7.5 y leche en polvo no grasa 5%, durante toda la noche a 4º C y se siguió el proceso descrito para el western blot de los PPAR hasta obtener la señal de la β-actina con el kit de quimioluminiscencia mencionado previamente. El anticuerpo secundario para el western blot de β-actina fue un anti-IgG de ratón conjugado con peroxidasa (Bio-Rad Laboratories, Hercules, CA, Estados Unidos de América) preparado en dilución 1:5000 disuelto en el mismo tampón utilizado para el anticuerpo primario. Para la β-actina se detectó una banda a 42 kDa.

La estimación cuantitativa de las proteínas de interés se realizó por densitometría, utilizando el sistema de análisis de imagen Quantity One (Bio-Rad Laboratories, Hercules, CA, USA). Los resultados obtenidos se expresaron en unidades arbitrarias.

3.5.6.1.- Determinación de proteínas totales

Las proteínas en los diferentes homogenizados obtenidos a partir de tejido hepático se determinaron mediante el método colorimétrico de Bradford con un reactivo comercial (Bio-Rad Laboratories, Hercules, CA, USA). Este método se basa en el cambio de absorbancia que en solución ácida presenta el colorante azul de Coomassie en respuesta a diferentes concentraciones de proteínas. La concentración de proteínas en la muestra es directamente proporcional a la absorbancia observada a una longitud de onda de 595 nm. Para la curva patrón se utilizó una solución de albúmina de 4.75 mg/ml, a partir de la cual se realizaron 6 diluciones mitad. Los resultados obtenidos se expresaron en mg/ml.

3.5.7.- Parámetros para determinar los niveles de TTR

La determinación de TTR se realizó en tejido hepático, para lo cual las muestras se homogenizaron a 4º C en un tampón de RIPA que contenía Tris-HCl 50 mM, NaCl 150 mM, SDS 0.1%, Deoxicolato de sodio 1% y Nonidet P40 1%, EDTA 5mM, Na3VO4 1 mM, NaF 50 mM, DTT 1 mM y una tableta del cóctel de inhibidores de proteasas Complete por cada 50 ml de tampón (Roche, Basilea, Suiza). Los homogenizados se centrifugaron a 12 000 g durante 10 min a 4º C, se separaron los sobrenadantes y se determinó la concentración de proteínas totales en los mismos tal y como se describe en el apartado correspondiente.

A continuación se procedió a realizar la técnica de western blot para la detección de TTR. Para esto se mezclaron alícuotas de los homogenizados con un tampón de carga de electroforesis que contenía β-mercaptoetanol y tampón Laemli en proporción 1:20. La proporción del homogenizado y el tampón de carga fue 1:1. Las proteínas se desnaturalizaron calentando la mezcla a 95ºC durante 10 min. Se cargaron 100 µg de proteína y se separaron las proteínas de acuerdo a su peso molecular por electroforesis en un gel de poliacrilamida/SDS al 12% a un voltaje constante de 56 mV en el Upper gel, y de 105 mV cuando las muestras se encontraban en el Lower gel. Posteriormente las proteínas fueron transferidas a una membrana de polifluoruro de vinilideno (PVDF) con una intensidad de 370 mA durante 70 minutos (30 min). Después de esto, los geles

se tiñeron con Comassie para asegurar que se había cargado igual cantidad de proteína y que las proteínas se habían transferido a la membrana de PVDF. Las membranas se incubaron en un tampón de bloqueo formado por una solución salina tamponada con Tris que contiene Tween-20 (TTBS) a pH=7.5 y leche en polvo no grasa al 3% durante 60 min a temperatura ambiente. A continuación las membranas se incubaron durante toda la noche a 4ºC con el anticuerpo primario policlonal anti-TTR (DAKO, Alemania) preparado en una dilución 1:300, disueltos en tampón de bloqueo. Al día siguiente, las membranas se lavaron con TTBS pH=7.5 y después se incubaron con el anticuerpo secundario durante 60 minutos a temperatura ambiente. En ambos casos se utilizó un anticuerpo secundario anti-IgG de conejo conjugado con peroxidasa (Bio-Rad Laboratories, Hercules, CA, USA) que fue preparado en dilución 1:2000 disuelto en tampón de bloqueo. Después de lavar la membrana con TTBS y finalmente con solución salina tamponada con Tris (TBS), se procedió a realizar la detección de las proteínas mediante el kit de quimioluminiscencia Immun-Star HRP (Bio-Rad Laboratories, Hercules, CA, USA) siguiendo las instrucciones del fabricante. Como marcador de peso molecular se utilizó el estándar preteñido Kaleidoscope (Bio-Rad Laboratories, Hercules, CA, USA) y se utilizó un control positivo para TTR (Sigma-Aldrich, España) que se analizó en paralelo a las muestras. Para TTR se detectó una banda a 14 kDa.

La estimación cuantitativa de las proteínas en las membranas se estandarizó respecto a la β-actina. Para ello las membranas se re-incubaron con un anticuerpo primario anti-β-actina de rata (Sigma-Aldrich, España) preparado en dilución 1:1000 disuelto en tampón que contenía TTBS pH=7.5 y leche en polvo no grasa 5%, durante toda la noche a 4º C y se siguió el proceso descrito para el western blot del PPAR hasta obtener la señal de la β-actina con el kit de quimioluminiscencia mencionado previamente. El anticuerpo secundario para el western blot de β-actina fue un anti-IgG de ratón conjugado con peroxidasa (Bio-Rad Laboratories, Hercules, CA, Estados Unidos de América) preparado en dilución 1:5000 disuelto en el mismo tampón utilizado para el anticuerpo primario. Para la β-actina se detectó una banda a 42 kDa.

La estimación cuantitativa de las proteínas de interés se realizó por densitometría, utilizando el sistema de análisis de imagen Quantity One (Bio-Rad Laboratories, Hercules, CA, USA). Los resultados obtenidos se expresaron en unidades arbitrarias.

3.5.8.- Parámetros para analizar el estrés oxidativo

Para evaluar el estrés oxidativo se determinaron los siguientes parámetros:

3.5.8.1.- Ensayo inmunoenzimático de Nitrotirosinas

Gran parte de los efectos perjudiciales del NO son mediados por el peroxinitrito, un producto de la reacción entre el NO y el superóxido, altamente oxidante y citotóxico. El peroxinitrito es una molécula muy inestable y altamente reactiva por lo que su producción es únicamente demostrable de forma indirecta, por ejemplo a través de los productos que forma. Por esto, la determinación de los niveles de nitrotirosinas se utiliza como marcador indirecto de la formación de peroxinitrito.

Para la determinación de los niveles de nitrotirosinas en el tejido hepático, las muestras se homogenizaron en 10 volúmenes de tampón Na2HPO4 50 mM pH=7.4 a 4º C, posteriormente se centrifugaron a 20 000 g durante 30 min a 4ºC, se recuperó el sobrenadante y se determinó en el mismo la concentración de proteínas totales siguiendo el protocolo descrito en el apartado correspondiente.

La cuantificación de los niveles de nitrotirosinas en el sobrenadante se llevó a cabo mediante un kit comercial inmunoenzimático (HyCult Biotech, Uden, Holanda) siguiendo las indicaciones del fabricante.

3.5.8.2.- Ensayo colorimétrico de MDA

El malondialdehído (MDA) es uno de los productos finales de la peroxidación lipídica y se utilizó como parámetro indirecto de estrés oxidativo (Baykal A., y cols. 1998). Los niveles de MDA se determinaron en muestras de hígado mediante el método del ácido tiobarbitúrico (TBA) (Ohkawa H., y cols 1979). En condiciones ácidas y a altas temperaturas el MDA reacciona con el TBA para dar lugar a un compuesto de color rosa, cuantificable por espectrofotometría.

Las muestras de tejido hepático congelado se homogenizaron en 2 ml de tampón Tris Base 0.1 M a pH=7 y se determinó la concentración de proteínas totales en el mismo, siguiendo el procedimiento descrito previamente en el apartado correspondiente. A 250 µl de este homogenado se le añadieron 250 µl de ácido tricloroacético (TCA) al 40% para precipitar las proteínas. Se mezclaron en un agitador tipo vórtex y se centrifugaron a 3000 rpm durante 15 min a 4ºC. Se recuperó el sobrenadante y se le añadió 250 µl de TBA 0.67% y se llevó a ebullición durante 15 min con lo que se desarrolló un color proporcional a la cantidad de MDA presente en las muestras.

Para la elaboración de la recta patrón, se preparó un estándar de MDA disolviendo 120 µl de 1,1,3,3-tetrahidroxipropano (Sigma-Aldrich, St. Louis, MO, USA) en 50ml de HCl 0.1M; esta solución se calentó durante una hora a 50ºC. Para la preparación de la recta de calibración se añadieron 50 µl de esta solución en 5 ml de agua. De esta solución así preparada, se tomaron 2.5 mL y se añadieron a 2.5 ml de agua, siendo éste el punto más concentrado de la recta patrón (50 nmoles/ml), a partir de ésta se hicieron 6 diluciones mitad. Como blanco se utilizó agua destilada. Los estándares y el blanco fueron sometidos al mismo procedimiento que las muestras a partir del tratamiento con TCA. Así pues, tras dejar enfriar, se determinó la intensidad de color midiendo la absorbancia a una longitud de onda de 530 nm. Los resultados se expresaron en nmol MDA/mg proteína.

3.6.- Estudio estadístico

El estudio estadístico se realizó mediante un análisis de la varianza (ANOVA), y seguidamente se determinó el nivel de significación estadística con un test Student-Newman-Keuls, considerando los datos significativamente diferentes cuando $p < 0.05$. Los datos están expresados como valor de la media ± error estándar de la media. Los estudios de dosis respuesta del RBP4 y del retinol se analizaron utilizando el programa GraphPad Prism versión 4 (GraphPad Software, San Diego, CA, USA).

4.- RESULTADOS

4.1.- Efectos del RBP4 en hepatectomía parcial con I/R

4.1.1.- Niveles de RBP4 en hígados esteatósicos y no esteatósicos

Los niveles proteicos y de mRNA del RBP4 en hígados esteatósicos y no esteatósicos sometidos a hepatectomía parcial con I/R fueron más bajos que en los grupos Sham (**Figura 18**).

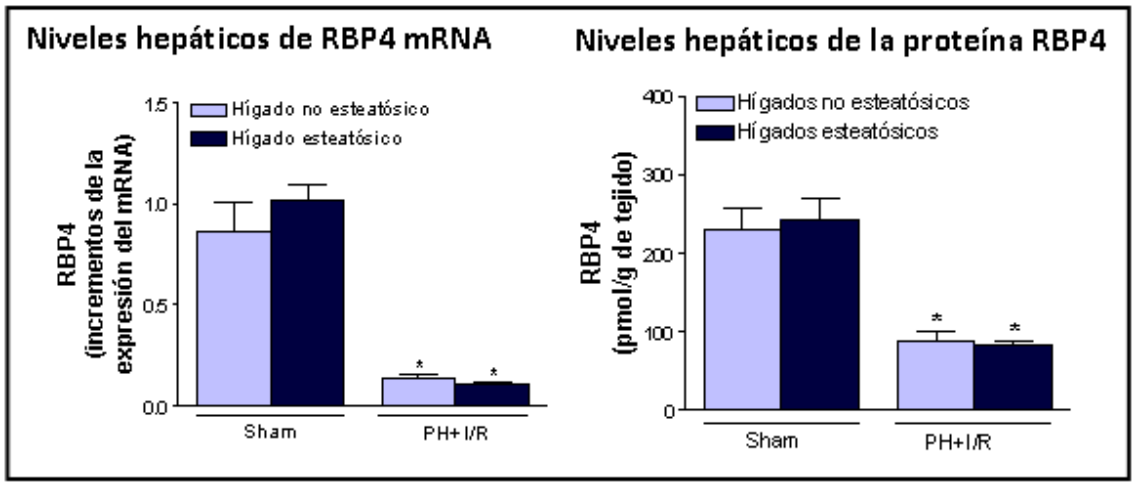


Figura 18. Niveles de RBP4 en hígados esteatósicos y no esteatósicos. Se evaluó la expresión génica del mRNA y los niveles de proteína del RBP4. *p < 0.05 versus grupo Sham.

4.1.2.- Daño hepático, regeneración y supervivencia

La administración de RBP4 disminuyó la supervivencia tanto en animales Ln como en Ob de forma directamente proporcional a la dosis administrada (**Figura 19**). En el caso de los animales Ob, la supervivencia a PH+I/R fue del 70%, mientras que la administración de RBP4 a dosis de 10 y 25 µg/kg redujo las tasas de supervivencia a 30% y 10% respectivamente. Todos los animales Ob con dosis de RBP4 de 50 y 150 µg/kg murieron durante los 2 primeros días después de la cirugía hepática (**Figura 19**). Se confirmó que la administración de RBP4 en dosis de 5 µg/kg no redujo la tasa de supervivencia, pero sí que agravó el daño hepático tanto en animales Ln como en Ob, aumentando el grado de lesión hepática y los niveles de transaminasas en comparación con los grupos PH+I/R (**Figura 20**). El número de hepatocitos Ki-67 positivos en ambos tipos de hígados fue menor al administrar RBP4 que en los grupos PH+I/R. Esta disminución de las células proliferativas se asoció con una bajada en los niveles de HGF y un aumento de TGF-β activa (**Figura 20**). Los niveles de TGF-β total fueron similares en todos los grupos. Las dosis de RBP4 inferiores a 5 µg/kg no se asociaron con la

protección hepática en ninguno tipo de hígado. Los efectos negativos del RBP4 no estaban relacionados con factores intrínsicos a la misma droga (por ejemplo: efectos secundarios tóxicos y la falta de especificidad). Esto se evidenció al administrar RBP4 en ratas Sham y no observar alteraciones en el daño hepático o en la regeneración. El análisis histológico reveló áreas severas, extensas y confluentes de necrosis coagulativa con infiltración de neutrófilos en los hígados esteatósicos tanto en el grupo PH+I/R como en el grupo RBP4. El grado de lesión hepática del grupo RBP4 fue significativamente mayor que el registrado en el grupo PH+I/R. Ambos grupos mostraron un porcentaje de hepatocitos Ki-67 positivos parecido (**Figuras 20 y 21**).

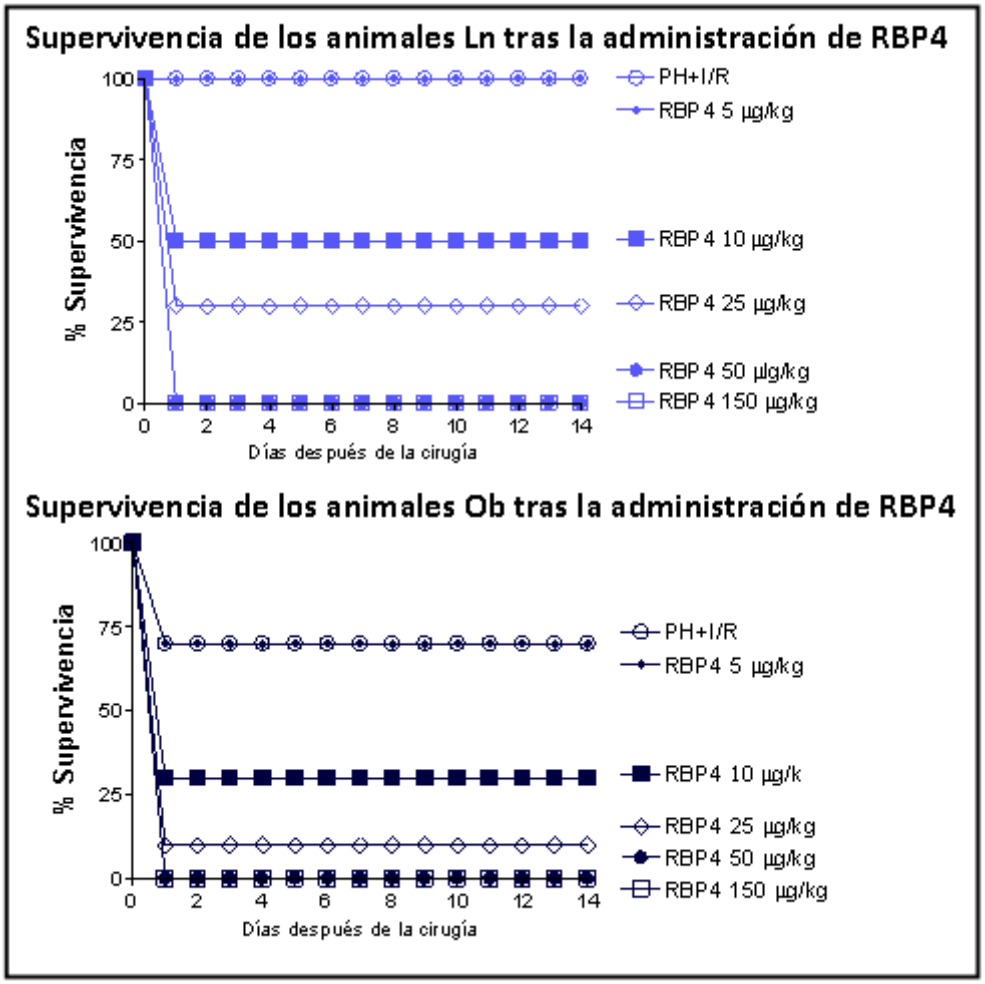


Figura 19. Supervivencia de las ratas esteatósicas y no esteatósicas sometidos a hepatectomía parcial, tras la administración de las distintas dosis de RBP4, a los 14 días después de la cirugía. Ratas con PH+I/R y sin administración de RBP4 (○), ratas con PH+I/R con administración de 5 µg/kg de RBP4 (♦), PH+I/R con administración de 10 µg/kg de RBP4 (■), PH+I/R con administración de 25 µg/kg de RBP4 (◊), PH+I/R con administración de 50 µg/kg de RBP4 (●), y PH+I/R con administración de 150 µg/kg de RBP4 (□).

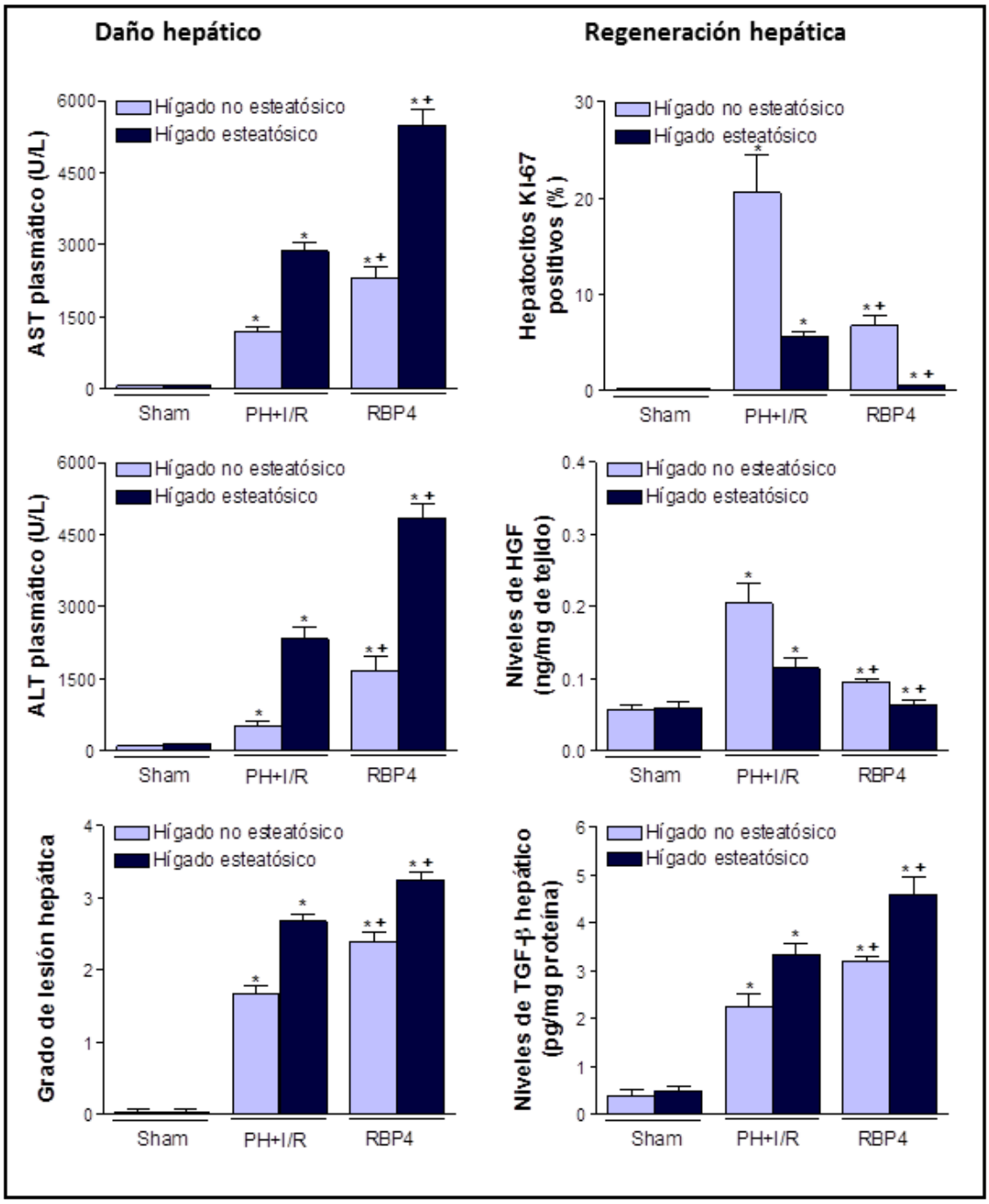


Figura 20. Efectos del RBP4 en el daño hepático (AST, ALT y grado de lesión hepática) y en la regeneración hepática (porcentaje de hepatocitos Ki-67 positivos, niveles de HGF y niveles de TGF-β) 24 horas después de la reperfusión. *p < 0.05 versus animales Sham, +p < 0.05 versus animales PH+I/R.

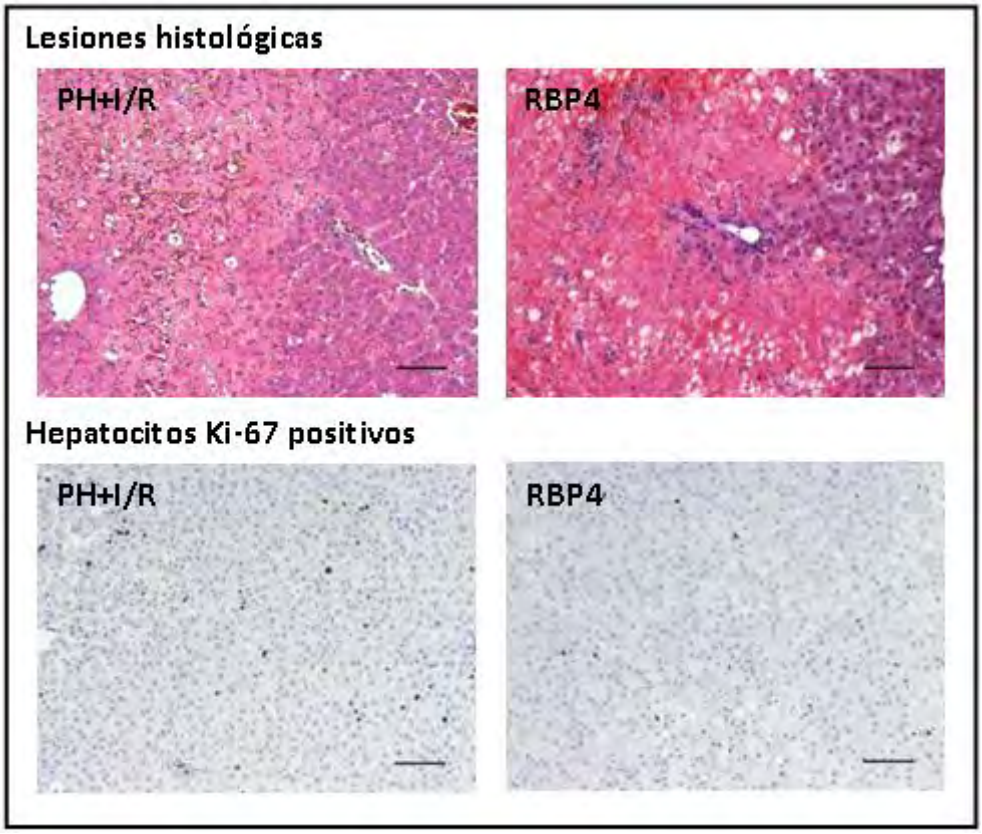


Figura 21. Estudio histológico de los efectos del RBP4 en hígados esteatósicos sometidos a hepatectomía parcial con I/R. Fotografías representativas con tinción de hematoxilina y eosina 24 horas después de la reperfusión. Se observan extensas áreas de necrosis coagulativa en ambos grupos (PH+I/R y RBP4). Fotografías representativas con inmunohistoquímica de hepatocitos Ki-67 positivos 24 horas después de la reperfusión. Se observan bajos niveles de hepatocitos Ki-67 positivos en ambos grupos experimentales (PH+I/R y RBP4). Barra = 1000 μm.

4.2.- Efectos del retinol en hepatectomía parcial con I/R

4.2.1.- Niveles de retinol en hígados esteatósicos y no esteatósicos

En hígados no esteatósicos, los niveles de retinol en el grupo PH+I/R fueron similares a los del grupo Sham. En cambio, las concentraciones de retinol en los hígados esteatósicos del grupo PH+I/R fueron menores a las del grupo Sham (**Figura 22**), lo que indica que la presencia de infiltración de grasa induce cambios en el metabolismo retinoico.

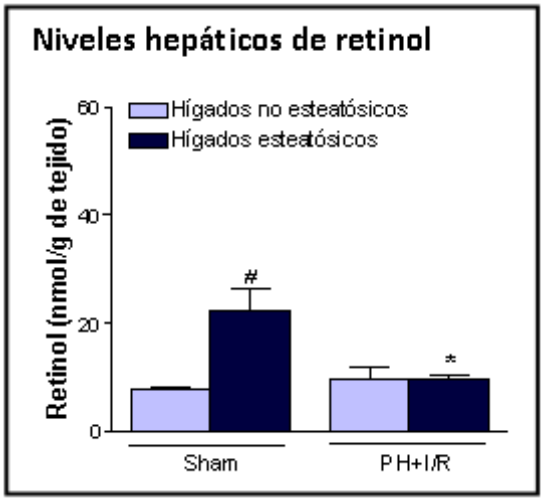


Figura 22. Niveles de retinol en hígados esteatósicos y no esteatósicos. *p < 0.05 versus grupo Sham, #p < 0.05 versus animales Ln.

4.2.2.- Daño hepático, regeneración y supervivencia

La administración de retinol redujo la supervivencia en los animales no esteatósicos de una manera dosis-dependiente. En estos animales, la administración de retinol a dosis de 10 y 15 mg/kg disminuyó las tasas de supervivencia a 70% y 30% respectivamente. Todos los animales Ln murieron en un plazo de 2 días después de la cirugía hepática cuando se administró retinol a una dosis de 30 mg/kg (**Figura 23**). Se confirmó que el retinol a una dosis de 5 mg/kg no redujo la tasa de supervivencia en animales Ln (**Figura 23**), pero afectó negativamente al daño y la regeneración hepáticos (ALT: 527,5 ± 73,9 y 225,6 ± 1720,5 en los grupos PH + I/R y PH+I/R+Retinol, respectivamente; valores de hepatocitos Ki-67 positivos: 20,6 ± 3,97 y 6,5 ± 1,2 en los grupos PH + I/R y PH+I/R+Retinol, respectivamente). Las dosis de retinol por debajo de 5 mg/kg no se asociaron con una protección hepática en hígados no esteatósicos.

Estos efectos del retinol son dependientes del tipo de hígado. En los animales esteatósicos la administración de retinol a dosis de 5 y 10 mg/kg aumentaron las tasas de supervivencia (90 y 100% respectivamente), en comparación con el grupo PH+I/R (70%) (**Figura 23**). Las dosis de retinol superiores a 10 mg/kg disminuyeron la supervivencia en los animales Ob a una tasa más baja que la observada en el grupo PH+I/R. La administración de retinol a dosis de 10 mg/kg en animales Ob (grupo Retinol) redujo los niveles de transaminasas y el grado de lesión hepática. La administración de retinol también aumentó el porcentaje de hepatocitos Ki-67 positivos en comparación con el grupo PH+I/R. Esta mejora se asoció con altos niveles de HGF y los niveles de TGF-b bajos (**Figura 24**). El análisis histológico reveló muy pocas áreas de necrosis en los hígados esteatósicos del grupo Retinol, en comparación con el grupo PH+I/R. El grado de lesión hepática del grupo Retinol fue significativamente menor que el registrado en el grupo PH+I/R. Además, en comparación con el grupo PH+I/R, el grupo Retinol exhibió un aumento del número de hepatocitos Ki-67 positivos en los hígados esteatósicos (**Figuras 24 y 25**).

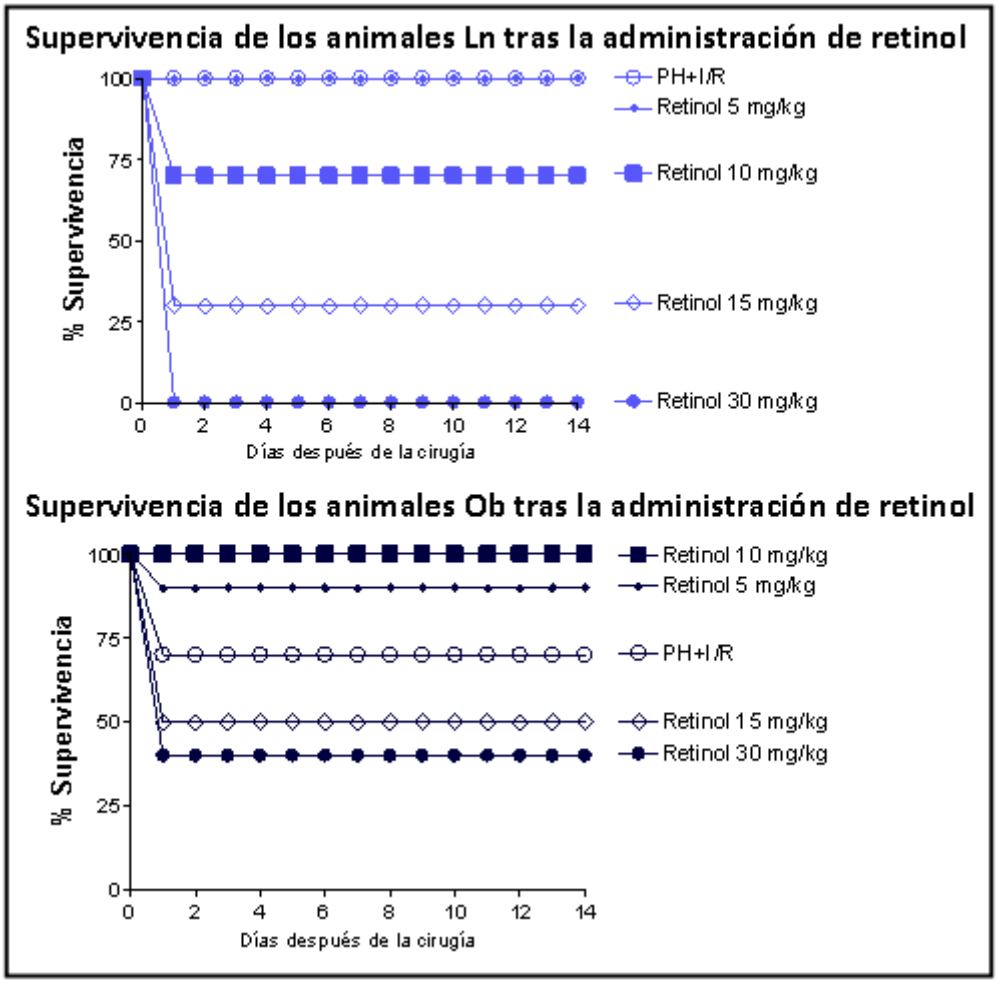


Figura 23. Supervivencia de las ratas esteatósicos y no esteatósicos sometidos a hepatectomía parcial con I/R, tras la administración de las distintas dosis de retinol, a los 14 días después de la cirugía. Ratas con PH+I/R y sin administración de retinol (○), ratas con PH+I/R con administración de 5 mg/kg de retinol (♦), PH+I/R con administración de 10 mg/kg de retinol (■), PH+I/R con administración de 15 mg/kg de retinol (◊), y PH+I/R con administración de 30 mg/kg de retinol (●).

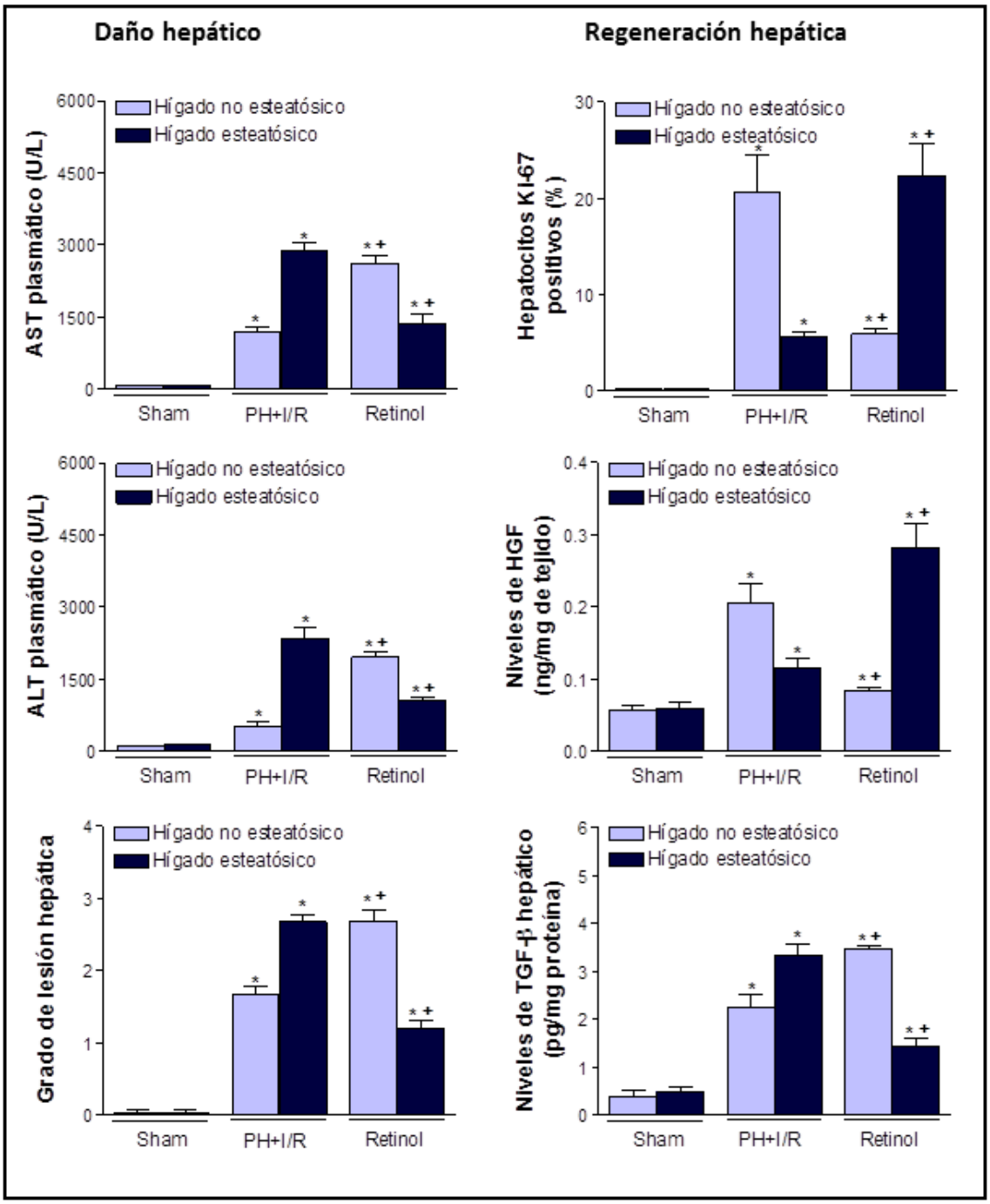


Figura 24. Efectos del retinol en el daño hepático (AST, ALT y grado de lesión hepática) y en la regeneración hepática (porcentaje de hepatocitos Ki-67 positivos, niveles de HGF y niveles de TGF-β) 24 horas después de la reperfusión. *p < 0.05 versus animales Sham, +p < 0.05 versus animales PH+I/R.

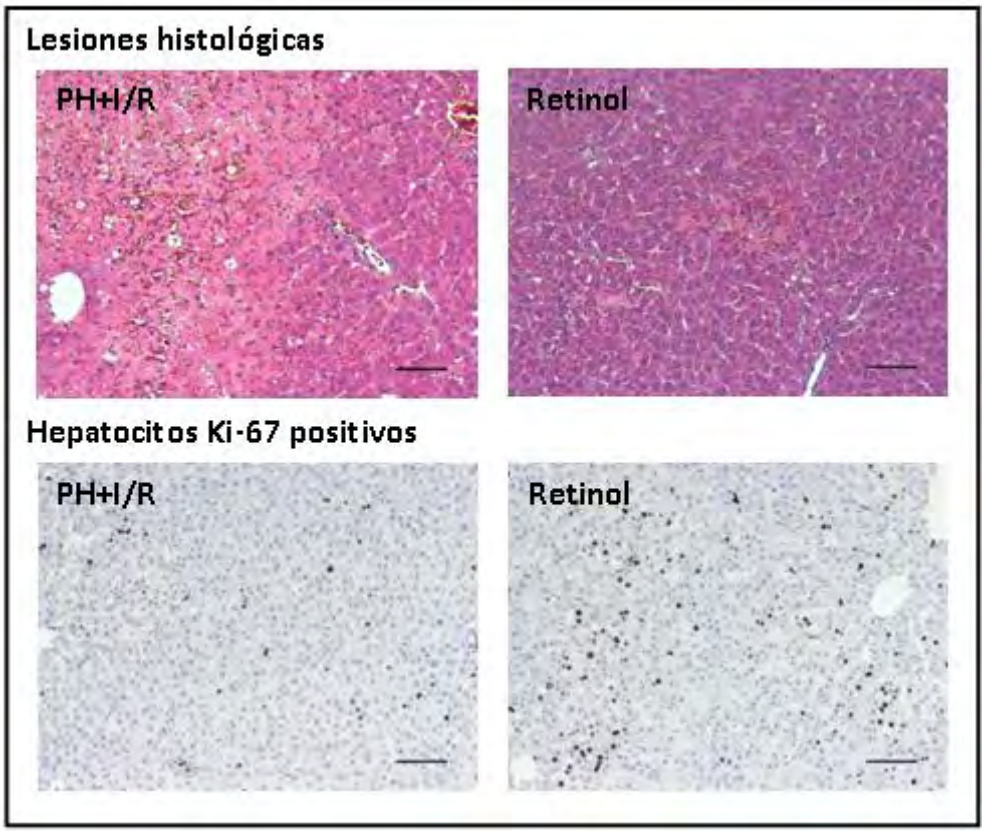


Figura 25. Estudio histológico de los efectos del retinol en hígados esteatósicos sometidos a hepatectomía parcial con I/R. Fotografías representativas con tinción de hematoxilina y eosina 24 horas después de la reperfusión. A diferencia del grupo PH+I/R, en los animales administrados con retinol se observan pequeñas áreas de necrosis coagulativa. Fotografías representativas con inmunohistoquímica de hepatocitos Ki-67 positivos 24 horas después de la reperfusión. A diferencia del grupo PH+I/R, en el grupo Retinol se observan altos niveles de hepatocitos Ki-67 positivos. Barra = 1000 μm.

4.3.- Efecto de RBP4 y retinol en combinación en hepatectomía parcial con I/R

En conjunto, los resultados presentados hasta el momento muestran que ni la administración de RBP4 ni de retinol protegen al hígado no esteatósico en condiciones de hepatectomía parcial con I/R. Una vez obtenidos los resultados de los efectos diferenciales del RBP4 y del retinol en el hígado esteatósico, y datos de la bibliografía indicando que cambio en RBP4 inducen cambios en los niveles de retinol (Gieng S.H., y cols. 2005; Rosales F.J., y cols. 1996), se evaluó si los efectos perjudiciales del RBP4 en daño y regeneración de hígados esteatósicos podrían explicarse por los cambios en los niveles de retinol. Para estos experimentos, se seleccionó la dosis menos perjudicial de RBP4 (5 mg/kg) y la dosis más efectiva de retinol (10 mg/kg).

4.3.1.- Daño hepático y regeneración

Administrados de forma individual, el RBP4 aumentó el daño en el hígado esteatósico, y el retinol lo disminuyó (**Figura 26**). Tal y como se ha comentado anteriormente, el RBP4 empeoró la regeneración hepática. Sin embargo, el retinol mejoró la regeneración de los hígados esteatósicos, como se indica por el aumento de HGF y los bajos niveles de TGF-β (**Figura 2**). La administración combinada de RBP4 y retinol (grupo RBP4+Retinol) en animales Ob dió lugar a parámetros de lesión y de regeneración hepáticos similares a los del grupo RBP4 (**Figura 26 y 27**).

El análisis histológico reveló áreas severas, extensas y confluentes de necrosis coagulativa con infiltración de neutrófilos en los hígados esteatósicos de los grupos PH+I/R, RBP4 y RBP4+Retinol (**Figura 27**). El grado de lesión hepática de los grupos RBP4 y RBP4+Retinol fueron significativamente mayores que los registrados para el grupo PH+I/R. Tanto el número como la extensión de las áreas necróticas en hígados esteatósicos se redujeron en el grupo Retinol. Además, en comparación con el grupo PH+I/R, el grupo Retinol exhibió un aumento del número de hepatocitos Ki-67 positivos en los hígados esteatósicos. Por lo contrario el número de hepatocitos Ki-67 positivos en los grupos RBP4 y RBP4+Retinol fue menor (**Figura 26 y 27**).

Por lo tanto, la administración de RBP4, por separado o en combinación con el retinol, afecta negativamente tanto el daño hepático como la regeneración.

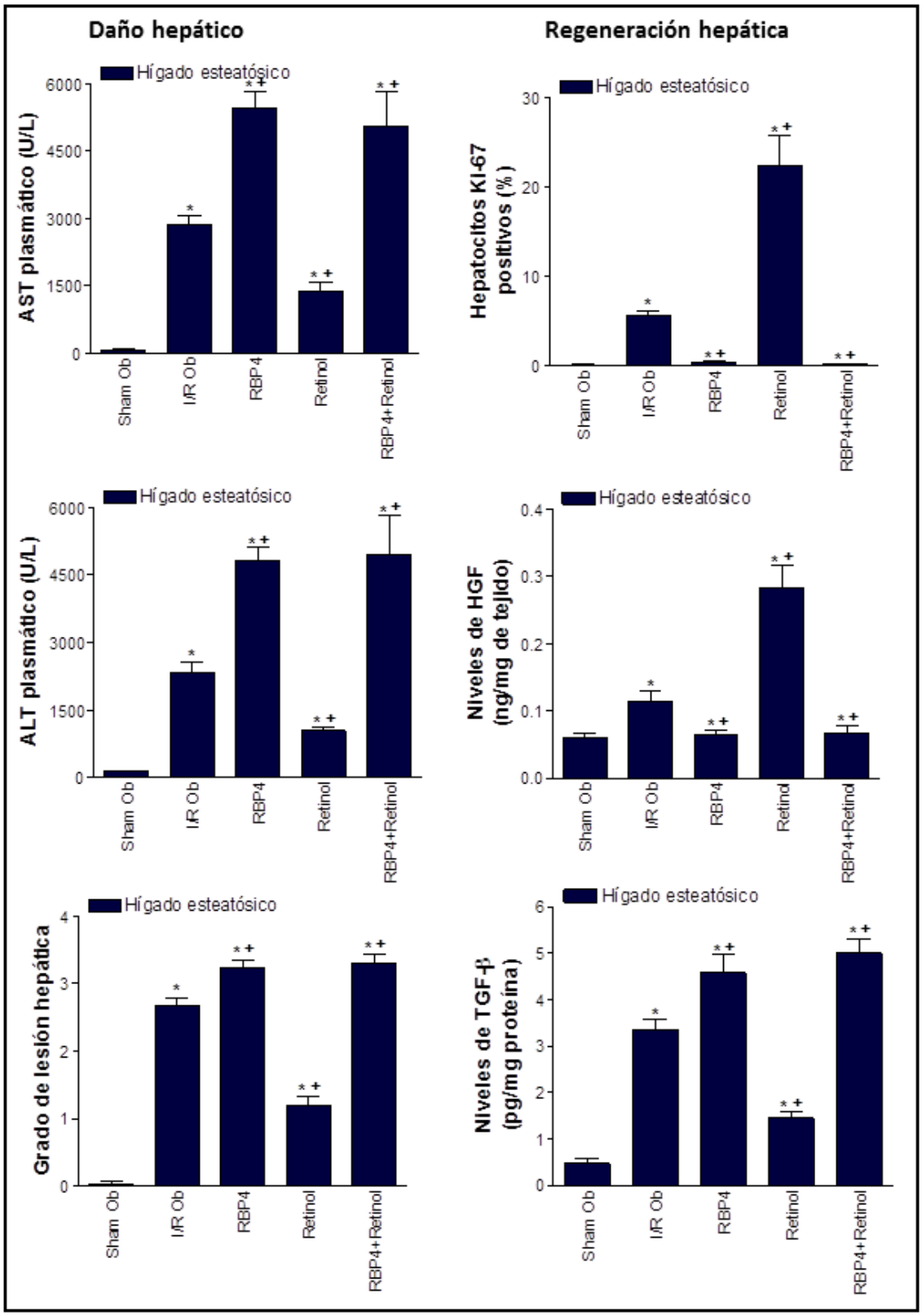


Figura 26. Efectos de la administración conjunta de RBP4 y retinol en el daño hepático (AST, ALT y grado de lesión hepática) y en la regeneración hepática (porcentaje de hepatocitos Ki-67 positivos, niveles de HGF y niveles de TGF-β) 24 horas después de la reperfusión. *p < 0.05 versus animales Sham, +p < 0.05 versus animales PH+I/R.

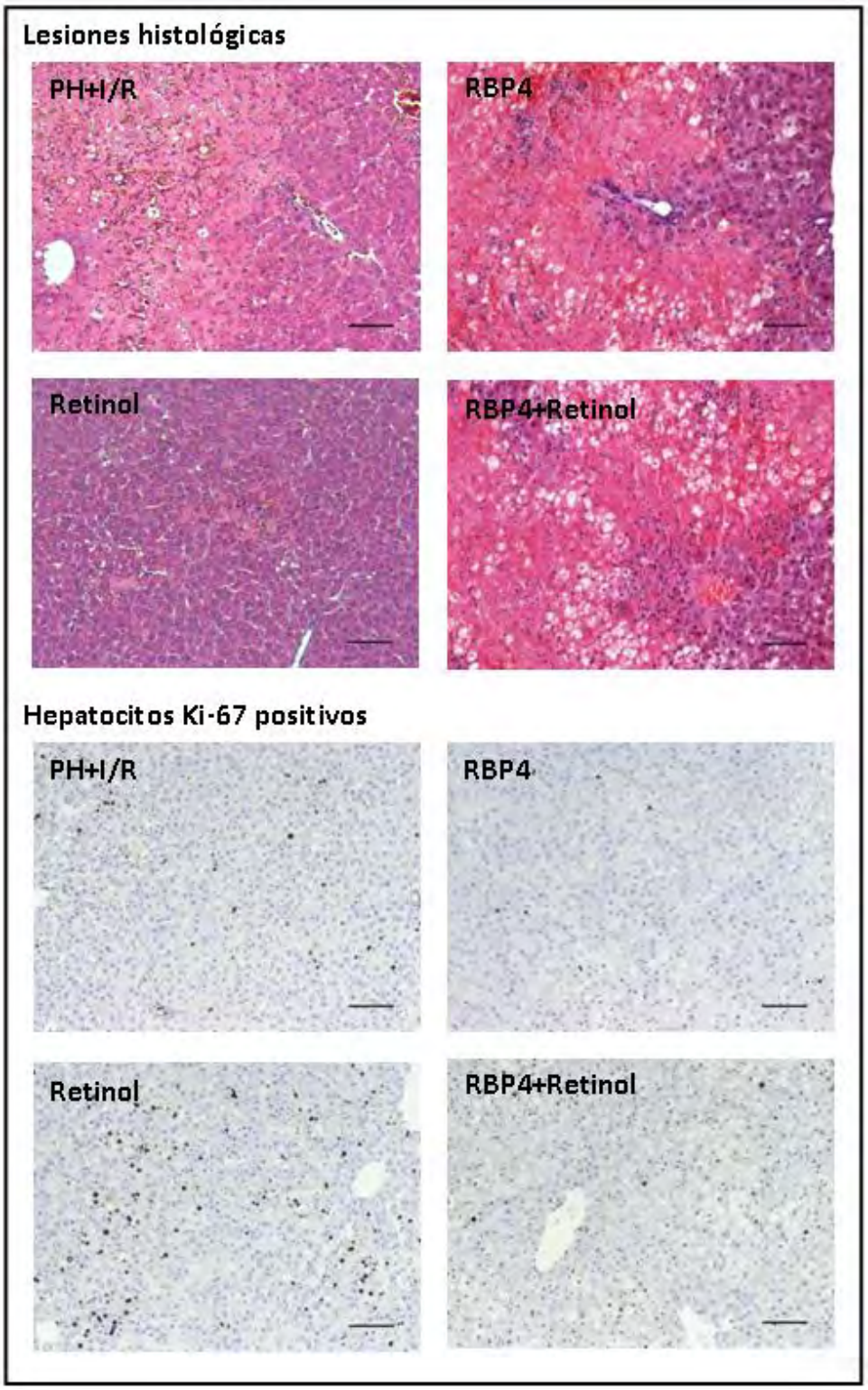


Figura 27. Estudio histológico de los efectos del retinol en las resecciones hepáticas parciales con I/R. Fotografías representativas con tinción de hematoxilina y eosina 24 horas después de la reperfusión. Se observan extensas áreas de necrosis coagulativa en los grupos PH+I/R, RBP4 y RBP4+Retinol; en cambio en el grupo Retinol estas son pequeñas. Fotografías representativas con inmunohistoquímica de hepatocitos Ki-67 positivos 24 horas después de la reperfusión. Se observan bajos niveles de hepatocitos Ki-67 positivos en los grupos PH+I/R, RBP4 y RBP4+Retinol; en cambio en el grupo Retinol estos son altos. Barra = 1000 μm.

4.3.2.- Ésteres de retinilo y retinol en hígado y plasma

Tal y cómo he mencionado anteriormente, la vitamina A se almacena en el hígado como ésteres de retinilo, y debe ser hidrolizada a retinol antes de ser movilizado en la circulación (Tuitoek P.J., y cols. 1996). En línea con esto, los niveles de ésteres de retinilo y retinol en hígados esteatósicos se redujeron en el grupo PH+I/R en comparación con el grupo Sam (**Figura 28A**). Esta disminución se asocia con altos niveles de retinol en plasma (**Figura 28B**). Como se sugirió anteriormente sobre la base de los estudios en células cultivadas (Sauvant P., y cols. 2001), el RBP4 podría afectar el almacenamiento y la movilización de retinol en los hígados esteatósicos. De hecho, se encontró que los niveles de ésteres de retinilo, pero no de retinol, en hígados esteatósicos se incrementaron mientras los niveles circulantes de retinol se redujeron en el grupo RBP4 en comparación con los grupos Sham y PH+I/R (**Figura 28B**). Como era de esperar, la administración de retinol (grupos Retinol y RBP4+Retinol) aumentó tanto los ésteres de retinilo como los niveles de retinol en los hígados esteatósicos en comparación con los observados en el grupo PH+I/R, y el aumento de los niveles de ésteres de retinilo, pero no de retinol, en el plasma (**Figura 28A y B**). Nuestros resultados confirmaron que la administración RBP4 indujo niveles más altos de RBP4 en animales Ob de los grupos RBP4 y RBP4+Retinol. Por ejemplo, los niveles de RBP4 hepáticos de los grupos RBP4+Retinol y PH+I/R eran 239,6 ± 21,65 y 82,41 ± 3,91, respectivamente ($P<0,05$). Los niveles hepáticos de RBP2 (pmol/g tejido) del grupo Retinol (83,45 ± 4,56) fueron similares a los correspondientes al grupo PH+I/R (82,41 ± 3,91, P=no significativo).

4.3.3.- Transporte de retinol en el plasma

Se observaron niveles plasmáticos reducidos de RBP4 en el grupo PH+I/R en comparación con el grupo Sham (**Figura 28C**). Los niveles plasmáticos de RBP4 detectados en los grupos RBP4 y RBP4+Retinol fueron menores que los del grupo PH+I/R. Por otro lado, los niveles plasmáticos de RBP4 en el grupo de Retinol fueron similares a los del grupo PH+I/R. Los niveles plasmáticos de TTR en el grupo PH+I/R se redujeron en comparación con el grupo Sham, mientras que los niveles de TTR en los grupos RBP4, Retinol y RBP4+Retinol fueron similares a los del grupo Sham. Se ha demostrado que el retinol se encuentra normalmente en el plasma en una relación molar 1:1 con RBP4 (Quadro L., y cols. 2003). En el grupo Sham la relación RBP4/Retinol

fue de aproximadamente 2; en los grupos PH+I/R y RBP4, la relación era mayor que 1, y en los grupos Retinol y RBP4+Retinol, la tasa era menor que 1 (**Figura 28C**).

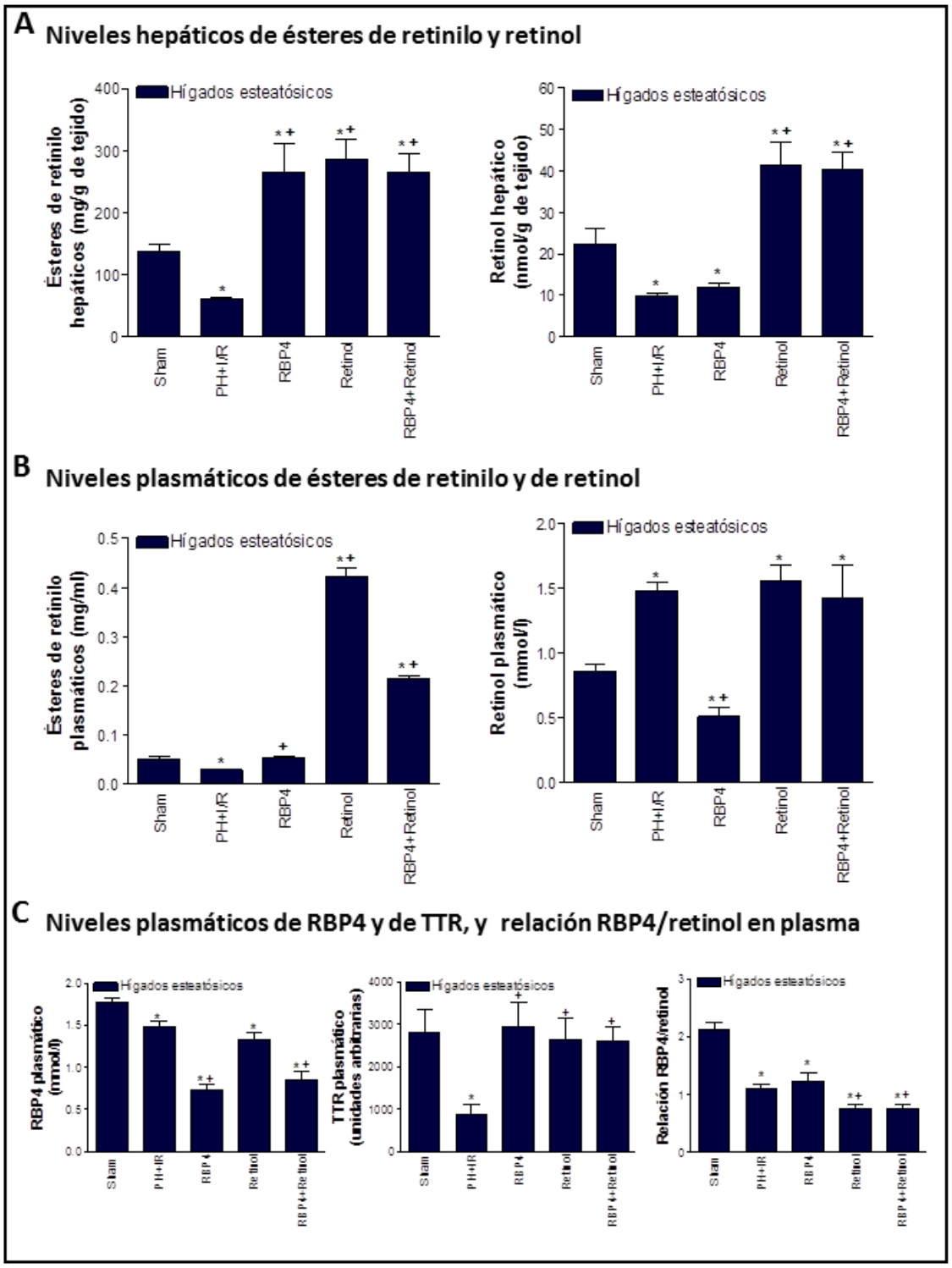


Figura 28. Efectos de la administración de RBP4 y de retinol (separados o combinados) en los niveles de ésteres de retinilo y retinol en (A) hígados esteatósicos y (B) en plasma de animales esteatósicos. El aparcado (C) muestra los niveles de RBP4 y TTR y en la relación RBP4/Retinol en plasma 24 horas después de la reperfusión. *p < 0.05 versus animales Sham, +p < 0.05 versus animales PH+I/R.

4.4.- Efecto del retinol sobre el daño hepático, la actividad proliferativa y el grado de esteatosis, en función del tiempo de reperfusión

Para estudiar los efectos del retinol dependiendo del tiempo de reperfusión se midió el daño hepático y la actividad proliferativa en los hígados esteatósicos y no esteatósicos a lo largo de la reperfusión (12, 24 y 48 horas) (**Figura 29**). En los animales Ln, los parámetros de lesión hepática a diferentes tiempos de reperfusión (RT) del grupo tratado con retinol, fueron mayores a los del grupo PH+I/R (**Figura 29A**). En los animales Ln, la administración de retinol deteriora el índice mitótico y la incorporación de BrdU hepatocelular después de la reperfusión. Por ejemplo, los porcentajes de hepatocitos BrdU positivos eran de 9,3% ± 1,09% y 19,5% ± 1,67% en los grupos Retinol y PH+I/R, respectivamente, 48 horas después de la reperfusión.

Por otro lado, en los animales Ob, los parámetros de daño hepático en los distintos tiempos de reperfusión fueron más bajos en el grupo Retinol en comparación con el grupo PH+I/R (**Figura 29B**). Nuestros resultados revelaron una mejora del proceso regenerativo en los hígados esteatósicos tratados con retinol en todos los tiempos de reperfusión, en comparación con el grupo PH+I/R. De esta forma, 48 horas después de la reperfusión, el porcentaje de hepatocitos BrdU positivos en el grupo Retinol fue del 17,3% ± 0,99%, mientras que sólo un 6,5% ± 0,76% de los hepatocitos del grupo PH+I/R eran BrdU positivos (**Figura 29A**).

Nuestros resultados sugieren una relación entre los efectos de la terapia del retinol y el grado de esteatosis en los animales Ob. Tal y como se ha comentado, Gazit V., y cols. (Gazit V., y cols. 2010) observaron un aumento de lípidos hepáticos durante la regeneración posterior a la hepatectomía parcial. En consonancia con los resultados observados a 24 horas de reperfusión, a 48 horas de reperfusión, también se observó un aumento de infiltración grasa hepatocelular y de triglicéridos tras la hepatectomía parcial con I/R (**Figura 30A y B**), mientras que en el grupo Retinol disminuyó. Los porcentajes de esteatosis fueron de 35,0% ± 1,78% y 83,13% ± 3,40% en los grupos Retinol y PH+I/R, respectivamente. La **Figura 31** muestra la relación entre los efectos de la terapia con retinol y el grado de esteatosis, a 12, 24, y 48 horas después de la reperfusión. Se observó una correlación directamente proporcional entre el grado de infiltración de grasa y los niveles de AST plasmática ($r = -0,9677$, $P<0,05$); y una

correlación inversamente proporcional entre el grado de infiltración de grasa y el índice mitótico ($r = -0,9724$, $P<0,05$). El tratamiento con retinol, por lo tanto, atenuó el daño hepático y mejoró la proliferación hepatocelular durante la regeneración del hígado en presencia de esteatósis. Esto se asoció con una acumulación reducida de grasa hepatocelular (**Figura 31**).

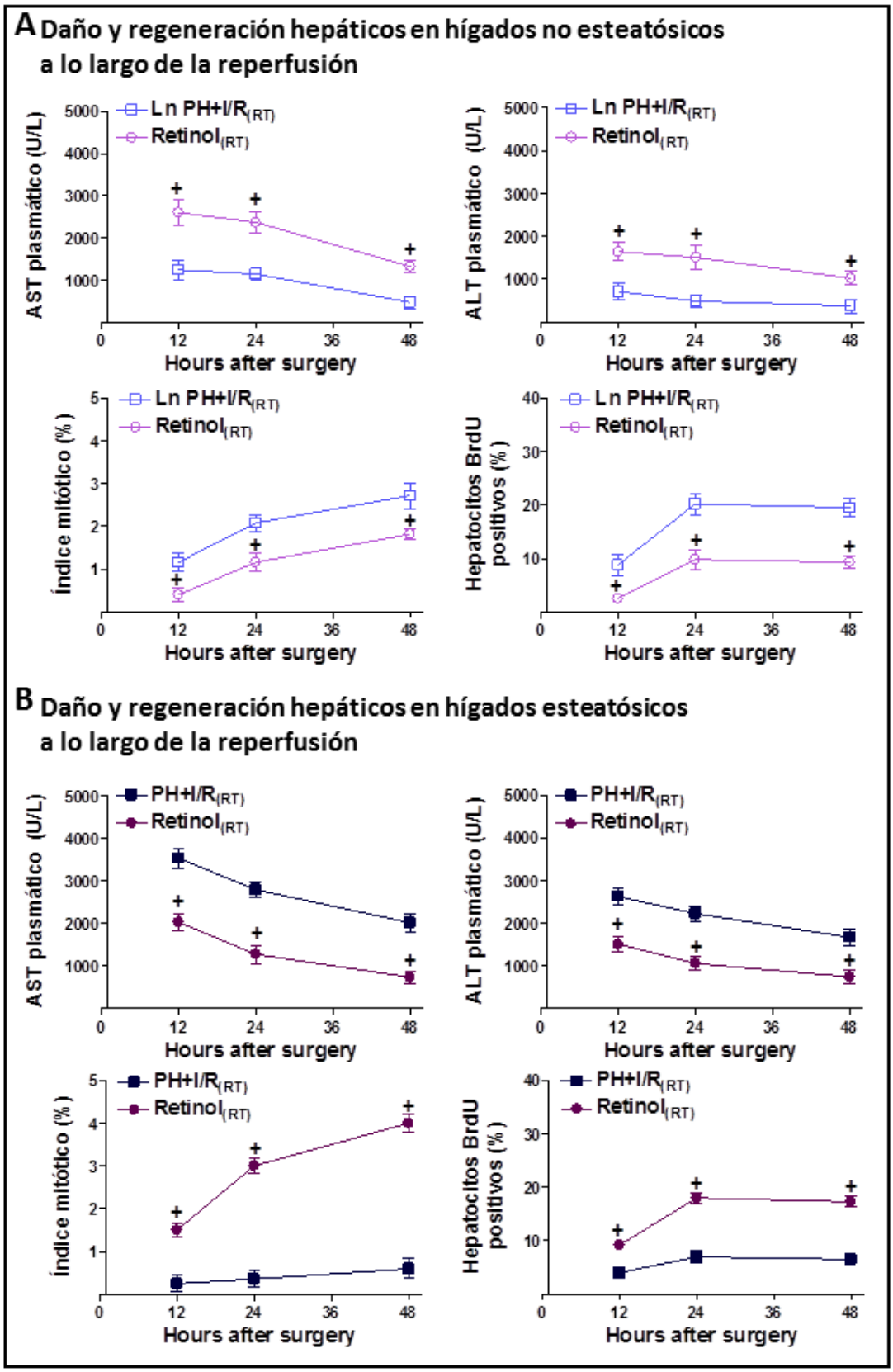


Figura 29. Efectos de la administración del retinol sobre el daño hepático (AST, ALT y grado de lesión hepática) y en la regeneración hepática (porcentaje de hepatocitos Ki-67 positivos, niveles de HGF y niveles de TGF-β) a lo largo de la reperfusión (12, 24 y 48 horas después de la reperfusión) en (A) hígados no esteatósicos y (B) hígados esteatósicos. +$p < 0.05$ versus animales PH+I/R.

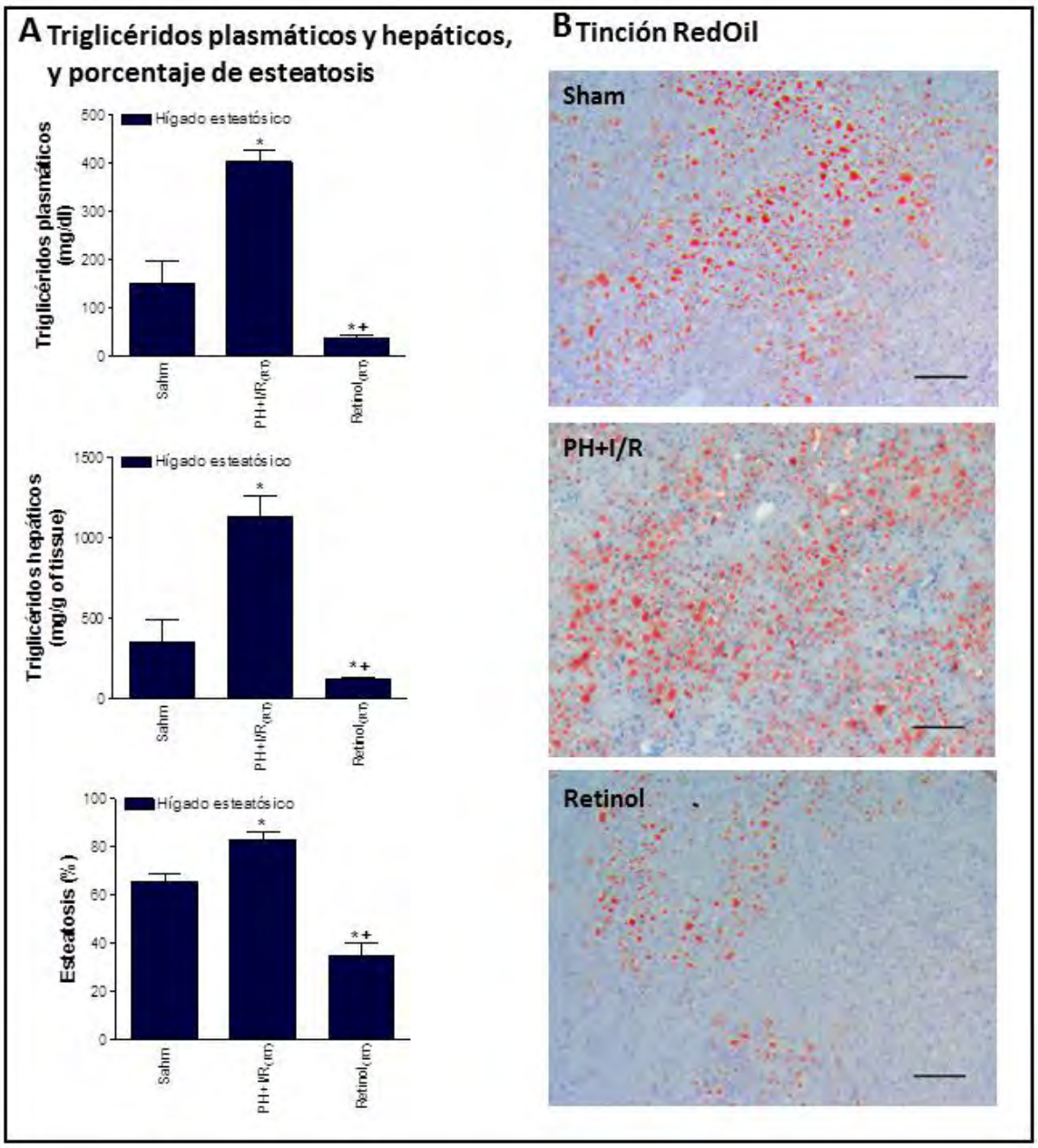


Figura 30. Efectos de la administración del retinol sobre (A) los niveles de triglicéridos plasmáticos y hepáticos, y el porcentaje de esteatosis. (B) Fotografías representativas de tinción RedOil a 48 horas de reperfusión. Los hígados esteatósicos del grupo Retinol mostraron menos glóbulos de grasa (color rojo) en los hepatocitos, en comparación con los hígados esteatósicos de los grupos Sham y PH+I/R. Barra = 1000 µm. *p < 0.05 versus animales Sham, +p < 0.05 versus animales PH+I/R.

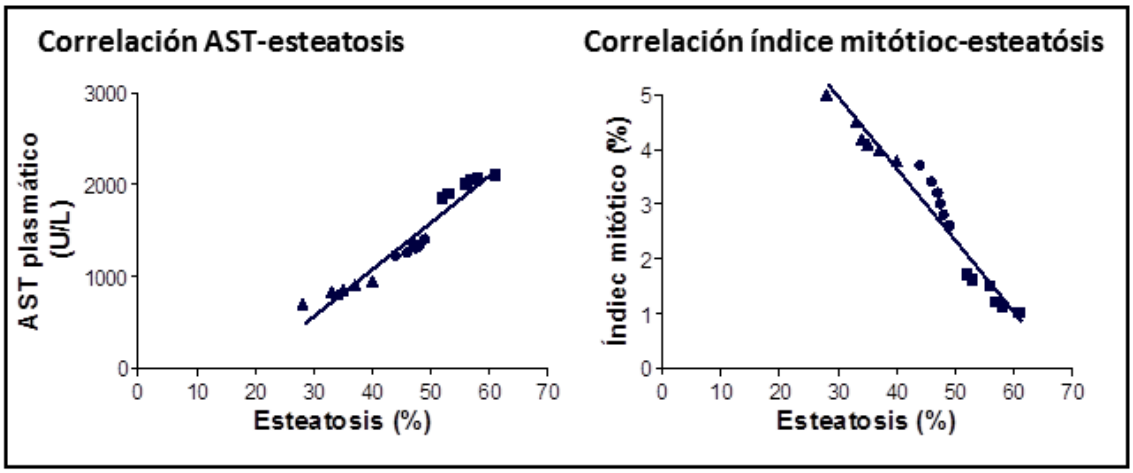


Figura 31. Relación entre el grado de esteatosis y (A) niveles de AST plasmático ($r=0.9677$, $P<0.05$) y (B) índice mitótico ($r=-0.9724$, $P<0.05$). (■) animales del grupo $Retinol_{(RT)}$ tras 12 horas de reperfusión, (●) animales del grupo $Retinol_{(RT)}$ tras 24 horas de reperfusión, y (▲) animales del grupo $Retinol_{(RT)}$ tras 48 horas de reperfusión.

4.5.- Mecanismo de acción del retinol en hepatectomía parcial con I/R

4.5.1.- El estrés oxidativo y los niveles de PPAR en el hígado esteatósico

Varios artículos sugieren que el retinol podría llevar a cabo sus funciones en los hígados esteatósicos mediante la reducción del estrés oxidativo y/o la activación de la transcripción de ciertos genes (Yang Q., y cols. 2005; Rao J., y cols. 2010; Oliveros L.B., 2007). En este estudio, los niveles de MDA y nitrotirosinas en el grupo de Retinol fueron similares a los detectados en el grupo PH+I/R (**Figura 30A**), lo que indica que el retinol no modificó el estrés oxidativo en hígados esteatósicos. Por otra parte, el retinol no alteró los niveles de PPAR-α peró si aumentó el PPAR-γ en comparación con el grupo PH+I/R (**Figura 32B**).

4.5.2.- Papel de PPAR-γ en los efectos beneficiosos de retinol en hígados esteatósicos

La inhibición del PPAR-γ en presencia de retinol (grupo Retinol+antagonista de PPAR-γ) eliminó los beneficios del retinol sobre el daño y la regeneración en los hígados esteatósicos. Los niveles de transaminasas y el grado de lesión hepática en el grupo Retinol+antagonista de PPAR-γ fueron similares a aquellos observados en el grupo PH+I/R (**Figura 33A**). El análisis histológico reveló áreas severas, extensivas y confluentes de necrosis coagulativa con infiltración de neutrófilos en los hígados esteatósicos del grupo Retinol+antagonista de PPAR-γ, las cuales fueron similares a las encontradas en el grupo PH+I/R. Tanto el número cómo la extensión de las áreas necróticas en los hígados esteatósicos se redujeron con la administración de retinol (**Figura 33B**). Sin embargo, las lesiones histológicas en el grupo Retinol+antagonista de PPAR-γ fueron similares a las del grupo PH+I/R. El grupo Retinol también exhibió un aumento del número de hepatocitos Ki-67 positivos en comparación con el grupo PH+I/R, lo que indica una mayor regeneración del hígado con la administración de retinol. Estos resultados se asociaron con altos niveles de HGF y bajos niveles de TGF-β (**Figura 34A**). Esto coincidió en una disminución del porcentaje de hepatocitos Ki-67 positivos y de los niveles de HGF, junto con un aumento en los niveles TGF- β, tanto en el grupo PH+I/R como en el grupo Retinol+antagonista de PPAR-γ (**Figura 34A y B**).

Conjuntamente, estos resultados sugieren fuertemente que los efectos beneficiosos del retinol están mediados por el PPAR-γ.

4.5.3 La acumulación de lípidos en el hígado esteatósico

Tras la hepatectomía parcial con I/R, se detecta un aumento de triglicéridos en hígado y plasma, e infiltración grasa hepatocelular (**Figura 35A y B**). Esto está en consonancia con un estudio anterior de Gazit y cols. (Gazit V., y cols. 2010) dónde observaron un aumento de lípidos hepáticos durante la regeneración posterior a la hepatectomía parcial. En este estudio, el retinol reduce esta acumulación de grasa hepática durante la regeneración hepática. Por otra parte, los niveles de triglicéridos y la infiltración grasa hepatocelular en el grupo Retinol+antagonista de PPAR-γ fue similar a la del grupo Retinol (**Figura 35A y B**), indicando que las acciones de retinol en este contexto son independientes del PPAR-γ.

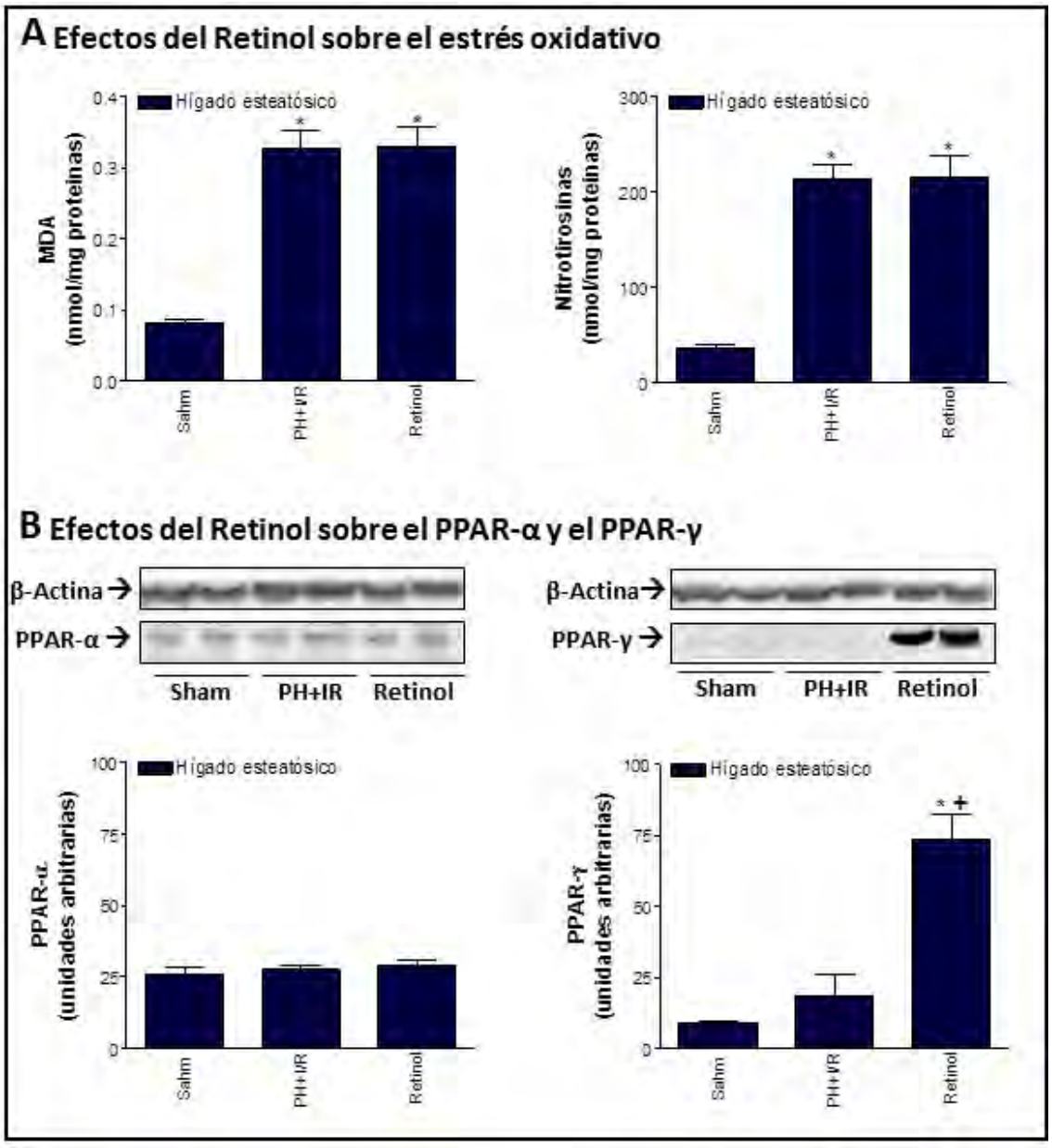


Figura 32. Efectos del retinol sobre (A) el estrés oxidativo y (B) PPAR-α y PPAR-γ, en hígados esteatósicos. (B) Encima, imágenes de western blot representativas de los niveles proteicos de PPAR-α y PPAR-γ; debajo, análisis densiométricos de los datos de los western blots. Las densidades de las bandas de PPAR-α y PPAR-γ fueron normalizadas con las de la β-actina. *p < 0.05 versus animales Sham, +p < 0.05 versus animales PH+I/R.

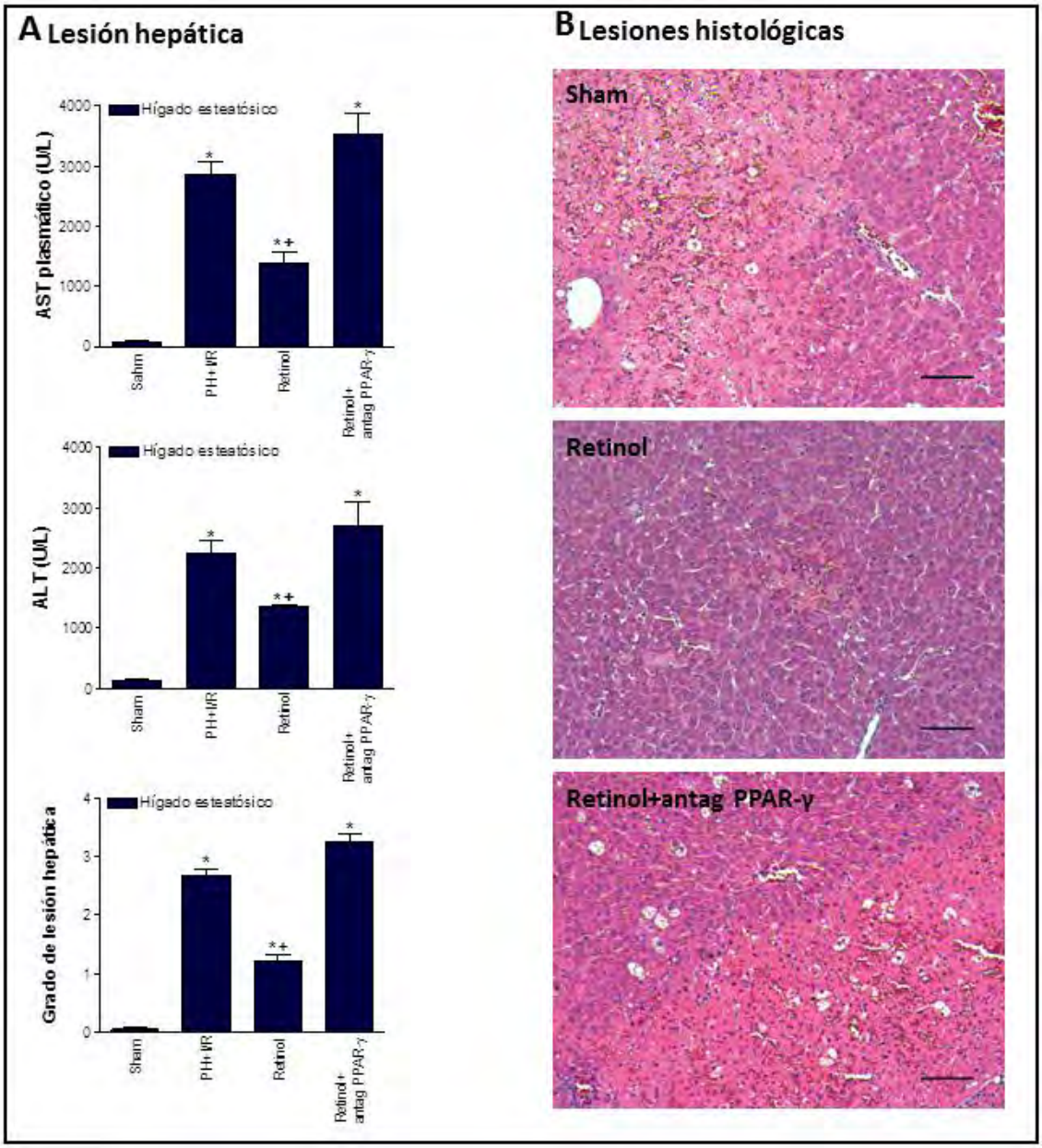


Figura 33. Papel del PPAR-γ en los efectos beneficiosos del retinol sobre la lesión hepática (transaminasas, grado de lesión hepática y fotografías histológicas representativas del daño hepático) en hígados esteatósicos. Las tinciones de hematoxilina-eosina muestran extensivas áreas de necrosis coagulativa en el grupo PH+I/R y pequeñas áreas en el grupo Retinol. Las lesiones hepáticas en el grupo Retinol+PPAR-γ antag son similares a las del grupo PH+I/R. Barra = 1000 μm. *$p < 0.05$ versus animales Sham, +$p < 0.05$ versus animales PH+I/R.

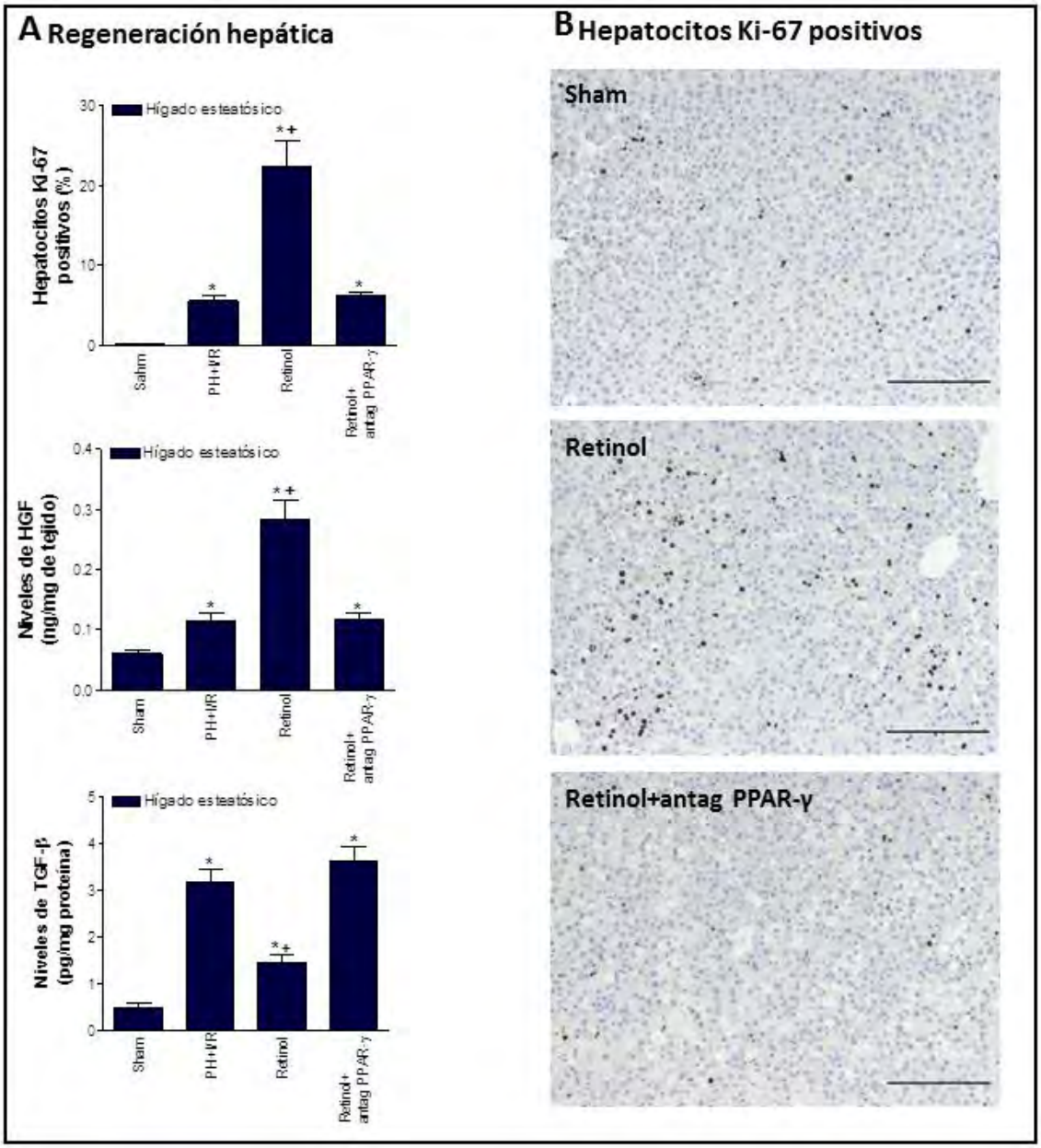


Figura 34. Papel del PPAR-γ en los efectos beneficiosos del retinol sobre la regeneración hepática (porcentaje de hepatocitos Ki-67 positivos, niveles de HGF y TGF-β y fotografías histológicas representativas de la inmunohistoquímica de hepatocitos Ki-67 positivos) en hígados esteatósicos. El porcentaje de hepatocitos Ki-67 positivos en el grupo PH+I/R y en el grupo Retinol+PPAR-γ antag fue menor que en el grupo Retinol. Barra = 1000 μm. *p < 0.05 versus animales Sham, +p < 0.05 versus animales PH+I/R.

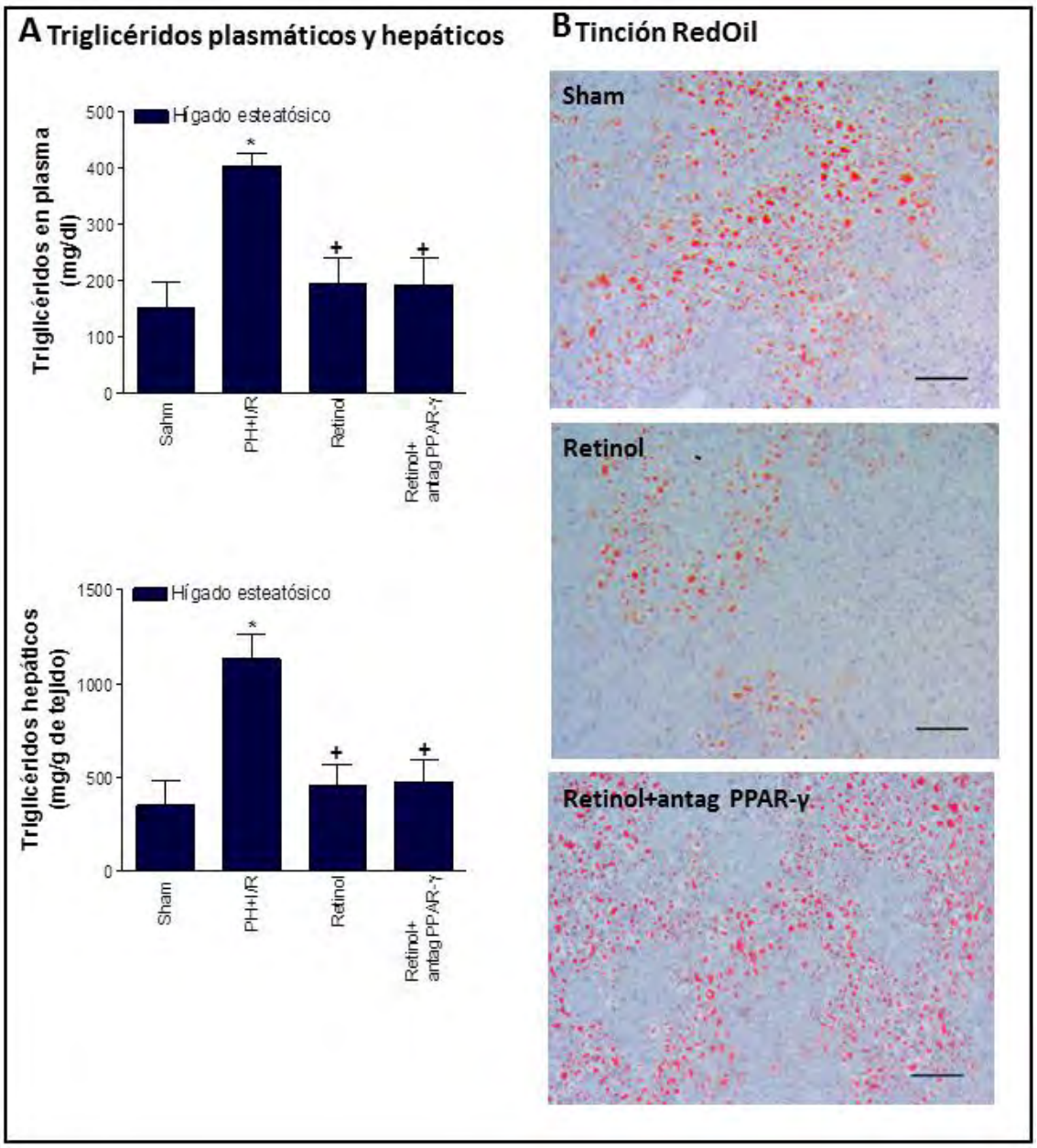


Figura 35. Efectos del retinol sobre (A) los niveles plasmáticos y hepáticos de triglicéridos. (B) Fotografías representativas de tinción RedOil. Los hígados esteatósicos del grupo Retinol mostraron menos glóbulos de grasa (color rojo) que los hígados de los grupos PH+I/R y Retinol+PPAR-γ antag. Barra = 1000 μm. *p < 0.05 versus animales Sham, +p < 0.05 versus animales PH+I/R.

5.- DISCUSIÓN

La lesión por I/R hepática, inherente a la cirugía hepática, continúa siendo un problema sin resolver en la práctica clínica a pesar de los recientes avances en los tratamientos farmacológicos, quirúrgicos y del establecimiento de estrategias de terapia génica. La I/R, usada para controlar el sangrado durante las resecciones hepáticas, está asociada a disfunciones hepáticas, siendo mayores los problemas operatorios en hígados esteatósicos por presentar poca tolerancia a la I/R (Ramalho F., y cols. 2006). En trasplante hepático, la I/R es la causa principal tanto del mal funcionamiento como del fallo primario del injerto hepático, este último responsable de 81% de los retrasplantes durante la primera semana tras la intervención quirúrgica. Y si esto ocurre en hígados sanos aun son mayores los casos de mal función o fallo primario del injerto hepático cuando el injerto es esteatósico, de ahí que la esteatosis sea la causa principal del mayor numero de órganos considerados no aptos para el trasplante, acentuando así la problemática de la falta de injertos hepáticos para trasplante (D'Alessandro A.M., y cols. 1995). Además, se espera una mayor prevalencia de esteatosis en la población en general y por lo tanto en la cirugía hepática (Veteläinen R., y cols, 2007). Teniendo en cuenta estas observaciones, es evidente la necesidad de desarrollar estrategias protectoras para minimizar los efectos adversos de la lesión por I/R en los hígados esteatósicos. Esto incidiría en una mayor disponibilidad de injertos hepáticos para trasplante y consecuentemente en una reducción en las listas de espera de pacientes que requieren un trasplante de hígado.

El RBP4 es una adipocitoquina que aumenta en obesidad y que puede tener propiedades pro-inflamatorias (Graham T.E. y cols. 2006; Yang Q., y cols. 2005; Cho Y.M., y cols. 2006; Lee D.C., y cols. 2007; Yao-Borengasser A., y cols. 2007). Se ha descrito que el RBP4 regula mediadores inflamatorios que también estarían implicados en la lesión por I/R hepática en hígados esteatósicos (Casillas-Ramírez A., y cols. 2006b; Graham T.E., y cols. 2006; Wu H., y cols. 2008). En la bibliografía solo existe un estudio sobre los efectos del RBP4 en I/R hepática. En este estudio, basado en un modelo de I/R hepática por transplante hepático, se sugirió que una modulación farmacológica del RBP4 protegía a los hígados esteatósicos frente a la lesión inducida por I/R fría. Teniendo en cuenta estas observaciones, y teniendo en cuenta la relevancia clínica de la I/R en resecciones hepáticas, se evaluó si la modulación farmacológica del RBP4 podría ser considerada como una estrategia protectora en hígados esteatósicos sometidos a una hepatectomía parcial del 70% con I/R.

En la presente tesis se evidencia que los niveles de RBP4 en hígados no esteatósicos sometidos a una resección parcial fueron prácticamente como el grupo Sham, pero se detectaron niveles de RBP4 reducidos en presencia de esteatosis. Este hallazgo está en consonancia con datos previos que han mostrado niveles reducidos de RBP4 en enfermedades hepáticas, tales como cirrosis, hepatitis aguda y malnutrición (Smith F.R., y cols. 1971; Smith J.C. Jr, y cols. 1975; McClain C.J., y cols. 1979); en diferentes tipos de inflamación inducida por lipopolisacárido o por IL-6 (Rosales F.J., y cols. 1996; Rosales F.J., y cols. 1998), e incluso en hígados esteatósicos sometidos a un trasplante (Casillas-Ramírez A., y cols. 2011). Esta disminución de los niveles de RBP4 observada en la circulación después de la hepatectomía parcial con I/R, podría reflejar una disminución de la síntesis hepática de RBP4 debido a una pérdida de tejido hepático funcional. Debido a que el hígado es el tejido principal de síntesis de RBP4 (Casillas-Ramírez A., y cols. 2011; Mody N., y cols. 2008), el daño de hepatocitos disminuye los niveles plasmáticos de RBP4. Por otra parte, la síntesis de proteínas de fase aguda negativas como RBP4 podría ser reducida en el hígado durante la inflamación o la cirugía como parte de una respuesta compensatoria al repentino aumento en la síntesis de proteínas de fase aguda positivas (Rosales F.J., y cols. 1996). Por otra parte, los efectos del RBP4 en I/R hepática podrían depender de las condiciones quirúrgicas. En efecto, mientras Casillas-Ramírez y cols. demostraron los efectos beneficiosos del RBP4 en hígados esteatósicos sometidos a trasplante hepático (Casillas-Ramírez A., y cols. 2011). La administración de RBP4 no solo no protege a los hígados esteatósicos y no esteatósicos en condiciones de hepatectomía parcial con I/R, sino que empeora la lesión hepática, el fallo en la regeneración y disminuye la tasa de supervivencia.

El retinol, que en condiciones normales se almacena en el hígado, se redujo en hígados esteatósicos tras hepatectomía parcial con I/R y aumentó en el plasma. Esto es consistente con el aumento de la secreción de retinol a partir de hígados esteatósicos en la circulación. Sin embargo, otras hipótesis propuestas en diferentes enfermedades hepáticas (Sato M., y cols. 1982), incluyendo el aumento del catabolismo de retinol en el hígado y/o la disminución de la captación hepática de ésteres de retinilo formado en los tejidos periféricos, no deben ser descartadas.

Siguiendo con los objetivos de la presente tesis, se estudió los efectos del RBP4 y la relación de este con el retinol en condiciones de resección hepática parcial con I/R. Estudios previos han descrito que en condiciones normales el retinol se encuentra en el plasma en una relación molar 1:1 con el RBP4 (la relación RBP4/retinol es

aproximadamente 1,0) (Soprano D.R., y cols. 1982). Por lo tanto, cuando la concentración de una de las moléculas aumenta, la otra molécula debe aumentar en la concentración para mantener la relación; de forma similar, las reducciones en una de ellas deben estar asociada con una reducción en la otra (Sato M., y cols. 1982). Sin embargo, en las condiciones experimentales de la presente tesis, y en consonancia con los datos de otros autores (Tuitoek P.J., y cols. 1996), la relación RBP4/retinol en el grupo de Sham de animales Ob (esteatósicos) fue aproximadamente de 2, lo que indica que la proporción de RBP4 en el plasma fue mayor que la de retinol. En este contexto, se debe considerar que RBP4 está presente tanto en holoforma (ligada al retinol) como en su apoforma (retinol-unido) y que RBP4 puede transportar y entregar otras moléculas lipofílicas además a retinol (Yang Q., y cols. 2005; Mallia A.K., y cols. 1975). Teniendo en cuenta que el TTR, tal como dice su nombre, transporta tiroxina y RBP4 unido al retinol en el plasma y en el líquido cefaloraquídedo, se estudió los niveles de TTR en los distintos grupos. En el grupo PH+I/R se observaron bajos niveles de RBP4 y de TTR. Esto estaría en consonancia con el hecho que el TTR también es una proteína de fase aguda negativa, y por lo tanto su síntesis también se ve reducida durante la inflamación (Rosales F.J., y cols. 1996). Al observar esta bajada en los niveles de RBP4 y TTR uno se podría preguntar cómo es que se mantienen los altos niveles plasmáticos de retinol en el grupo PH+I/R. Sin embargo, a pesar del descenso en los niveles de RBP4 y TTR, la relación RBP4/retinol en este grupo continúa siendo mayor que 1, y por lo tanto hay suficiente RBP4 para unirse al retinol. De esta misma forma, se puede suponer que los niveles reducidos de TTR en plasma del grupo PH+I/R también son suficientes para mantener el complejo retinol-RBP4 en plasma, ya que los niveles circulantes de TTR están, por lo general, 3-5 veces en exceso molar a los del RBP4 circulante (Rosales F.J., y cols. 1996; Mody N., y cols. 2008). Esta falta de correlación entre los niveles de RBP4 y de TTR en plasma, pese a ser las dos proteínas de fase aguda negativas y tener una menor síntesis durante la inflamación (Rosales F.J., y cols. 1996), podría explicarse, al menos en parte, por una eliminación diferencial por los riñones (Mody N., y cols. 2008). El TTR, que tiene un peso molecular de 14 kDa, se encuentra en la circulación en forma de homotetrámero, y por lo tanto tiene un peso molecular final de 56 kDa. Por otro lado, el RBP4 tiene un peso molecular de 21 kDa. De esta forma, la unión del RBP4 a la TTR, le permite resistir a la filtración glomerular y reducir su eliminación renal (Mody N., y cols. 2008). Por otra parte, el TTR, que es sintetizado en el hígado y en el plexo coroideo, tiene funciones adicionales, como por

ejemplo la de transportar la hormona T4 (Tuitoek P.J., y cols. 1996). Y por lo tanto, sus niveles vendrían regulados por su relación con el RBP4, y por su relación con la hormona T4.

Posteriormente se evaluó el efecto de la administración conjunta de RBP4 y retinol. El RBP4, que como se ha comentado anteriormente tiene efectos perjudiciales sobre la lesión por I/R, también ejerce efectos perjudiciales sobre la regeneración hepática en ambos tipos de hígado. Al administrar conjuntamente el RBP4 y el retinol, se observó que estos efectos perjudiciales del RBP4 son independientes del retinol. Por otra parte, los cambios en el metabolismo del retinol inducidos por el RBP4 deben ocurrir simplemente como consecuencia de la progresión de la lesión hepática por I/R, y por lo tanto no podría explicar los mecanismos de los efectos perjudiciales del RBP4. El mecanismo que explica estos hallazgos no está claro. Los ésteres de retinilo parecen estar afectados por la administración de RBP4, sugiriendo que el RBP4 tiene acciones independientes al simple transporte de retinol. Sin embargo, este aumento en los niveles de ésteres de retinilo inducido por el RBP4 no explica los efectos perjudiciales del RBP4 en este modelo experimental. En efecto, la pérdida de retinoides, en lugar de un aumento de los niveles de retinoides, es la que contribuye al desarrollo de enfermedades hepáticas (Blaner W.S., y cols. 2009). Se ha sugerido que la reducción en los niveles de retinoides observada en pacientes con enfermedad hepática alcohólica no podía ser debida a una malnutrición, pero podría ser debida a un aumento de la degradación de retinoides en el hígado o a un aumento de la movilización de estos hacia tejidos extrahepáticos. Pero en los resultados de la presente tesis no solo no se observó un aumento de retinoides plasmáticos al tratar las ratas con RBP4, sino que disminuían sus niveles plasmáticos. De esta manera, no es probable que en estas condiciones experimentales los retinoides sean movilizados a otros tejidos. De esta forma, se podría esperar que los tejidos extrahepáticos que dependen del retinol como fuente de vitamina A se vieran afectados por los bajos niveles plasmáticos de retinol inducidos por el RBP4. Sin embargo, esto tampoco explicaría los efectos perjudiciales del RBP4 en el hígado. En efecto, cuando el RBP4 se administra en combinación con el retinol la tasa de supervivencia de los animales es baja, y similar a la observada cuando se administra solo RBP4.

En estudios previos a la presente tesis se detectaron elevados niveles de RBP4 plasmáticos en ratones resistentes a insulina y en humanos con obesidad y diabetes tipo 2. Un aumento en la expresión de RBP4 por transgénesis o por inyección de RBP4

recombinante en ratones normales causa resistencia a insulina. Por otra parte, una deleción genética del RBP4 incrementa la sensibilidad a la insulina (Yang Q., y cols. 2005). Estudiar si el RBP4 también tiene efectos metabólicos no era uno de los objetivos de la presente tesis. Sin embargo, debido a los resultados obtenidos, esto no parece ser el caso del modelo experimental usado. Las ratas Zucker obesas utilizadas en la presente tesis tienen el receptor de la leptina mutado, y, como resultado, son hiperfágicas, obesas y hiperinsulinemicas. Estas ratas, con resistencia a la insulina, pero con niveles normales de glucosa en sangre no desarrollan diabetes. Las ratas Zycker Lean mantienen su fenotipo Lean durante su vida, con niveles normales de insulina y glucosa en sangre. La administración de RBP4 no alteró ni la insulina ni la glucosa plasmáticas en las ratas Zucker Ob sometidas a PH+I/R, y por lo tanto no había relación alguna entre los niveles de RBP4 y la resistencia a insulina. En efecto, las ratas Zucker Ln y Ob del grupo Sham mostraron niveles de RBP4 similares en hígado y plasma, mientras que solo las ratas Zucker Ob son insulina-resistentes. Hay que tener en cuenta, en el estudio antes mencionado (Lanne B., y cols. 2006), tanto los ratones genéticamente Ob, como los ratones Ob inducidos dietéticamente con resistencia a insulina, exhibieron mayores niveles de RBP4 plasmáticos en comparación con los controles Ln. Las diferencias en los niveles plasmáticos de RBP4 observados entre el mencionado estudio y la presente tesis pueden ser debidos, al menos en parte, a diferencias en la regulación del RBP4 entre ratones y ratas (Lanne B., y cols. 2006). Todo esto indica que en las condiciones de la presente tesis el RBP4 no afecta la resistencia a la insulina.

Futuros estudios, que no forman parte de los objetivos de la presente tesis, son necesarios para entender porqué la modulación farmacológicas del RBP4 ejerce estos efectos dañinos en los hígados sometidos a resección parcial con I/R. Es posible que cambios compensatorios en la síntesis proteica de proteínas de fase aguda positivas y negativas, las cuales son necesarias para restaurar la homeostasis proteica tras una resección hepática, fueran perturbados en el hígado restante. Desde un punto de visto clínico, estrategias basadas en la modulación de RBP4 no deben ser apropiadas en resecciones hepáticas o en condiciones quirúrgicas como el trasplante de injertos hepáticos de tamaño reducido. De hecho al administrar RBP4 para compensar su reducción tras la cirugía, se observaba un empeoramiento de la lesión y la regeneración hepáticas.

Posteriormente se evaluó el efecto del retinol. A diferencia de la administración de RBP4, el pretratamiento con retinol podría abrir nuevas posibilidades para intervenciones terapéuticas en la resección de hígados esteatósicos. Tal y como se esperaba, la administración de retinol aumentó el almacenaje de vitamina A en el hígado. El aumento de los niveles plasmáticos de ésteres de retinilo, pero no de retinol o RBP4, tras la administración de retinol sugiere que el retinol es incorporado en los hepatocitos como ésteres de retinilo y secretado junto con lipoproteínas. Por lo tanto, la administración de retinol podría suministrar vitamina A a los tejidos diana. Teniendo esto en cuenta, junto con estudios que evidencian que el RBP4 solo contendría un sitio de unión a retinol (Mody N., y cols. 2005), el hecho de que la relación RBP4/retinol tras la administración de retinol sea menor que 1 sugiere que una fracción de retinol está unido a transportadores distintos al RBP4, tal y como ocurre en otras patologías (Mallia A.K., y cols. 1975). Se he observado que el retinol unido a RBP4 no parece manifestar sus efectos en las membranas biológicas (Dingle J.T., y cols. 1972). Por lo tanto, futuros estudios clínicos basados en los beneficios del retinol, deberían centrarse en los posibles transportadores de retinol en estas condiciones. También se debería estudiar si estos efectos beneficiosos del retinol están activos durante el transporte, pues se ha observado que el modo de transporte de retinol puede ser un determinante importante en el desarrollo de las manifestaciones de hipervitaminosis A.

Por otro lado, en este modelo experimental, y independientemente de cual o cuales puedan ser el transportador que ayuda al RBP4 a transportar el retinol que se encuentra en exceso, los resultados presentados en la presente tesis indican que el retinol reduce el daño y mejora la regeneración del hígado en un modelo experimental PH+I/R. Resultados previos a la presente tesis indicaron que la terapia con retinol afectó a hígados esteatósicos sometidos tanto a I/R, como a hepatectomía parcial. Por otro lado, el retinol reduce el daño hepático en ratas Ob sometidas a isquemia parcial de 1 hora y sin hepatectomía; y reduce el daño hepático y el fallo regenerativo de hígados esteatósicos sometidos a resección parcial del 70%.

Estudios previos han demostraron que el ácido al-trans-retinoico protegía los hígados no esteatósicos contra los efectos dañinos del estrés oxidativo en isquemia caliente sin hepatectomía (Rao J., y cols. 2010). En las condiciones de la presente tesis, de hepatectomía parcial del 70% con I/R, el retinol solo protegía los hígados esteatósicos, y lo hacía por un mecanismo independiente del estrés oxidativo. Estas diferencias entre el estudio de Rao y cols., y la presente tesis podrían ser debidas a diferencias en el fármaco

usado, la dosis y frecuencia del fármaco administrado, y a diferencias de las condiciones quirúrgicas. En las condiciones de la presente tesis el retinol disminuía la lesión hepática, aumentaba la regeneración tras la hepatectomía y reducía los niveles de esteatosis.

Se evaluó el PPAR-α y el PPAR-γ como posibles mediadores implicados en los beneficios del retinol debido a que estudios previos habían apuntado hacia una relación entre los PPAR y el retinol (Yang Q., y cols. 2005; Oliveros L.B., y cols. 2007). Los resultados demostraron que a diferencia con lo publicado anteriormente, el retinol no modificaba los niveles de PPAR-α. Por el contrario, la administración de retinol sobrerreguló los niveles de PPAR-γ para proteger los hígados esteatósicos de la lesión por I/R y del fallo de la regeneración hepáticas tras la cirugía. En contraste con el papel clave del PPAR-γ en estas acciones protectoras del retinol, los efectos del retinol sobre la acumulación de lípidos fueron independientes de PPAR-γ. De esta manera, la sobrerregulación de PPAR-γ en condiciones quirúrgicas de PH+I/R en hígados esteatósicos protegería dichos hígados; mientras que en condiciones de trasplante hepático una inhibición de PPAR-γ protegería a los hígados esteatósicos.

Futuros estudios son necesarios para conocer cuáles son los mecanismos por los cuales el retinol previene la acumulación de lípidos en el hígado regenerativo tras la cirugía. Los efectos del retinol sobre la movilización de los ácidos grasos de los tejidos periféricos, la lipogénesis hepática, la oxidación hepática de los ácidos grasos, y los mecanismos de secreción de triglicéridos hepáticos deben ser explorados como posibles respuestas a esta pregunta. Por otra parte, se deben llevar a cabo investigaciones para determinar si estos cambios en la acumulación lipídica hepática inducidos por el retinol son relevantes para el daño y la regeneración hepáticos. De acuerdo con resultados previos, el pre-tratamiento con adiponectina disminuye la esteatosis hepática mediante la regulación a la baja de FAS y UCP-2 e incrementa los niveles de ATP, mejorando la regeneración post-quirúrgica (Elias-Miró M., y cols. 2011). Por otro lado, estrategias como la regulación adiponectina, que reducen la esteatosis hepática, deberían considerarse con cautela antes de aplicarse a la práctica clínica, ya que otros estudios, principalmente con hígados no esteatósicos, han demostrado que estrategias basadas en la reducción de la acumulación de lípidos hepáticos podría empeorar la regeneración post-quirúrgica. Se sabe que durante la regeneración hepática hay una acumulación transitoria de grasa en el hígado procedente del tejido adiposo para suministrar la energía necesaria al hígado. Por lo tanto, las estrategias basadas en la interrupción de

esta esteatosis transitoria deberían considerarse con mucha cautela, ya que dependiendo del estado inicial del hígado (esteatósicos o no esteatósicos) tendría efectos completamente distintos. Teniendo en cuenta esto, una pregunta a resolver sería cuánto se debe reducir la esteatosis en los hígados esteatósicos para proteger este tipo de hígados; ya que como se ha mencionado anteriormente, una reducción en el contenido de grasa hepática en los hígados no esteatósicos podría estar asociada con una alteración de la regeneración, debido a la falta de gotas lipídicas que le suministren la energía necesaria para la regeneración hepática. Otro punto importante por resolver es si esta esteatosis debería ser reducida antes de la intervención quirúrgica, y, por lo tanto, evitar la vulnerabilidad de los hígados esteatósicos a la I/R, o utilizar medicamentos para reducir los niveles de triglicéridos hepáticos durante la cirugía y así conservar la energía necesaria para la regeneración del hígado.

Desde el punto de vista de una posible aplicación clínica, los hallazgos descritos en la presente tesis pueden abrir nuevas posibilidades para la intervención terapéutica en las resecciones hepáticas con I/R. Mientras el RBP4 en estas condiciones no debería ser modulado farmacológicamente, el retinol sería una diana terapéutica apropiada en los hígados esteatósicos sometidos a una resección parcial con I/R. Así pues, una administración de retinol recombinante, o de agonistas de retinol podría ser efectiva en las hepatectomías parciales con I/R en hígados esteatósicos, reduciendo así el daño asociado a la lesión por I/R y aumentando la regeneración hepática a través de la sobrerregulación del PPAR-γ. Estas estrategias no serían útiles en ausencia de esteatosis hepática. Además, estrategias dirigidas a reducir la esteatosis de los hígados esteatósicos, ya sea a través del retinol u otro mecanismo como la adiponectina, podrían ser útiles para mejorar la regeneración post-quirúrgica. Aun y así, y tal y como se ha comentado anteriormente, se debería estudiar a partir de qué porcentaje de esteatosis son útiles estas estrategias, y cuánta esteatosis es necesaria reducir para poder observar estos beneficios regeneratorios.

6.- CONCLUSIONES

Las conclusiones de la presente tesis son las siguientes:

1. En condiciones de hepatectomía parcial con I/R, los hígados esteatósicos y no esteatósicos presentaron niveles de RBP4 más bajos que los hígados de ratas Sham.
2. El RBP4 está implicado en la lesión por I/R y en el fallo en la regeneración hepática asociados a las hepatectomías parciales con I/R en ambos tipos de hígados.
3. La modulación farmacológica del RBP4, empeora la regeneración hepática y reduce la supervivencia en ambos tipos de hígado sometidos a cirugía.
4. Los datos obtenidos respecto a la ratio RBP4/retinol, indican que el RBP4 puede transportar otras moléculas diferentes al retinol.
5. Los efectos perjudiciales del RBP4 son independientes de retinol. Los cambios en el metabolismo del retinol inducidos por el RBP4 ocurren como consecuencia de la progresión de la lesión y del fallo en la regeneración.
6. Los ésteres de retinilo están afectados por el RBP4, indicando que el RBP4 tiene acciones independientes al simple transporte del retinol.
7. Después de un proceso de hepatectomía parcial con I/R, los hígados esteatósicos presentaron niveles de retinol inferiores a los hígados esteatósicos Sham, mientras que los hígados no esteatósicos no mostraron ningún cambio en sus niveles de retinol tras la cirugía.
8. La administración de retinol aumenta la lesión, empeora la regeneración y reduce la supervivencia en hígados no esteatósicos.
9. Por otro lado, la administración de retinol en los hígados esteatósicos tiene efectos beneficiosos sobre la lesión, la regeneración y la supervivencia tras la cirugía hepática.
10. La relación RBP4/retinol en hígados esteatósicos sometidos a resección hepática parcial con I/R y pre-tratamiento con retinol está por debajo de 1, y por lo tanto una parte de este retinol plasmático está transportado por algún transportador, desconocido, diferente del RBP4.
11. Los beneficios del retinol en la resección hepática parcial con I/R no ocurren a través de una mejora en el estrés oxidativo. Los efectos beneficiosos del retinol en la lesión por I/R y la regeneración hepáticas están mediados por un aumento en la expresión del PPAR-γ y una reducción de la esteatosis hepática. Este

aumento del PPAR-γ no es responsable de los efectos beneficiosos del retinol sobre la reducción de la esteatosis hepática.

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ANEXOS

ANEXO 1

En este primer anexo se especifican y adjuntan los artículos que forman parte de la presente Tesis:

1. **Elias-Miró M***, Massip-Salcedo M*, Raila J, Schweigert F, Mendes-Braz M, Ramalho F, Jiménez-Castro MB, Casillas-Ramírez A, Bermudo R, Rimola A, Rodes J, Peralta C. Retinol binding protein 4 and retinol in rat steatotic and non-steatotic livers in partial hepatectomy under ischemia-reperfusion. Liver Transpl 2012 Oct;18(10):1198-208. *Ambos autores contribuyeron igualmente al artículo.

2. **Elias-Miró M***, Massip-Salcedo M*, Raila J, Schweigert F, Mendes-Braz M, Ramalho F, Jiménez-Castro MB, Casillas-Ramírez A, Bermudo R, Rimola A, Rodes J, Peralta C. The beneficial effects of Retinol in steatotic livers undergoing liver surgery are mediated by PPAR-γ. Contribución al congreso EASL del 2013. (Amsterdam, Holanda). (Artículo en preparación) *Ambos autores contribuyeron igualmente al artículo.

3. **Elias-Miró M**, Massip-Salcedo M, Jiménez-Castro M, Peralta C. Does adiponectin benefit steatotic liver transplantation? Liver Transpl 2011 Sep;17(9):993-1004.

4. **Elias-Miró M**, Jiménez-Castro MB, Mendes-Braz M, Casillas-Ramírez A, Peralta C. The Current Knowledge of the Role of PPAR in Hepatic Ischemia-Reperfusion Injury. PPAR Research 2012;2012;802384.

5. **Elias-Miró M**, Jiménez-Castro MB, Peralta C. The current knowledge of the oxidative stress in hepatic ischemia-reperfusion. Free Radical research. 2013;47(8):555-568.

6. **Elias-Miró M***, Jiménez-Castro MB*, Peralta C. Ischemia-Reperfusion Injury Associated with Liver Transplantation in 2011: Past and Future. Capítulo del libro: Liver transplantation - Basic issues. InTech, ISBN: 978-953-51-0016-4. *Ambos autores contribuyeron igualmente al artículo.

7. **Elias-Miró M**, Jiménez-Castro MB, Peralta C. The role of adenosine triphosphate in marginal and normal liver grafts undergoing transplantation. NOVA Science Publishers 2012, ISBN: 978-1-62417-891-7 Adenosine Triphosphate: Chemical Properties, Biosynthesis and Functions in Cells.

LIVER TRANSPLANTATION 18:1198-1208, 2012

ORIGINAL ARTICLE

Retinol Binding Protein 4 and Retinol in Steatotic and Nonsteatotic Rat Livers in the Setting of Partial Hepatectomy Under Ischemia/Reperfusion

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Steatotic livers show increased hepatic damage and impaired regeneration after partial hepatectomy (PH) under ischemia/reperfusion (I/R), which is commonly applied in clinical practice to reduce bleeding. The known function of retinol-binding protein 4 (RBP4) is to transport retinol in the circulation. We examined whether modulating RBP4 and/or retinol could protect steatotic and nonsteatotic livers in the setting of PH under I/R. Steatotic and nonsteatotic livers from Zucker rats were subjected to PH (70%) with 60 minutes of ischemia. RBP4 and retinol levels were measured and altered pharmacologically, and their effects on hepatic damage and regeneration were studied after reperfusion. Decreased RBP4 levels were observed in both liver types, whereas retinol levels were reduced only in steatotic livers. RBP4 administration exacerbated the negative consequences of liver surgery with respect to damage and liver regeneration in both liver types. RBP4 affected the mobilization of retinol from steatotic livers, and this revealed actions of RBP4 independent of simple retinol transport. The injurious effects of RBP4 were not due to changes in retinol levels. Treatment with retinol was effective only for steatotic livers. Indeed, retinol increased hepatic injury and impaired liver regeneration in nonsteatotic livers. In steatotic livers, retinol reduced damage and improved regeneration after surgery. These benefits of retinol were associated with a reduced accumulation of hepatocellular fat. Thus, strategies based on modulating RBP4 could be ineffective and possibly even harmful in both liver types in the setting of PH under I/R. In terms of clinical applications, a retinol pretreatment might open new avenues for liver surgery that specifically benefit the steatotic liver. ***Liver Transpl 18:1198-1208, 2012.*** © **2012 AASLD.**

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In clinical situations, partial hepatectomy (PH) under ischemia/reperfusion (I/R) is usually performed to control bleeding during parenchymal dissection.[1] Hepatic steatosis, a major risk factor for liver surgery, is associated with an increased complication index and increased postoperative mortality after major liver

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BrdU, bromodeoxyuridine; HGF, hepatocyte growth factor; I/R, ischemia/reperfusion; Ln, lean; mRNA, messenger RNA; Ob, obese; PH, partial hepatectomy; RBP4, retinol-binding protein 4; TGF-β, transforming growth factor β; TTR, transthyretin; $I/R_{(RT)}$, ischemia/reperfusion at different reperfusion times.

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resection.[1] In comparison with nonsteatotic livers, steatotic livers show impaired regenerative responses and reduced tolerance to hepatic damage.[2]

Retinol-binding protein 4 (RBP4) is an adipokine synthesized by the liver; its known function is to transport retinol in the circulation.[3] The mobilization of liver vitamin A, which is stored predominantly as retinyl esters, requires the hydrolysis of retinyl esters to free retinol.[4,5] The retinol-RBP4 complex is secreted into the circulation, in which it binds transthyretin (TTR). The association of RBP4 with TTR stabilizes the complex in the circulation. Upon the delivery of retinol to target cells, RBP4 loses its affinity for TTR and is then eliminated through the kidneys.[4,5] It should be considered that RBP4 is not merely a transport protein for retinol. Indeed, RBP4 directly exerts injurious effects in several pathologies, including diabetes and cardiovascular diseases.[3,6,7] However, the administration of RBP4 has been reported to be beneficial against I/R damage in steatotic liver transplantation.[8]

In the present study, we examined whether the modulation of RBP4 could protect steatotic and/or nonsteatotic livers against damage and regenerative liver failure after PH under I/R. Because of the central importance of RBP4 in the homeostatic regulation of retinol,[9] we evaluated whether changes in RBP4 levels induced by PH under I/R could affect the circulating and tissue levels of retinol. Indeed, the accumulation of retinol in the liver during inflammation and the low plasma retinol levels observed in different pathologies have been attributed to a decrease in hepatic RBP4 synthesis.[10,11] We also investigated the role of retinol in nonsteatotic and steatotic livers in the setting of PH under I/R, which is commonly applied in clinical practice to reduce blood loss. To the best of our knowledge, only 1 experimental study of hepatic I/R (notably focused on nonsteatotic livers without hepatectomy) has reported that a derivative of retinol (all-trans retinoic acid) protects against I/R damage.[12] Some studies of PH without vascular occlusion that have focused on nonsteatotic livers have reported apparently controversial effects of retinol or its derivatives on hepatic regeneration.[13,14] A greater understanding of the role of retinol in the setting of PH under I/R could contribute to the development of new pharmacological strategies for hepatic resections.

MATERIALS AND METHODS

Experimental Animals

Male, homozygous, obese (Ob) Zucker rats (400-450 g) and male, heterozygous, lean (Ln) Zucker rats (350-400 g; Iffa Credo, France) that were 14 to 16 weeks old were used in these experiments. The Ob Zucker rats showed severe macrovesicular and microvesicular fatty infiltration in hepatocytes (60%-70% steatosis), whereas the Ln Zucker rats showed no evidence of steatosis. This study complied with European Union regulations on animal experiments (directive 86/609/EEC).

Surgical Procedure

The experiments in this study employed a rat model of PH (70%) with 60 minutes of ischemia, as previously described.[15] Briefly, after anesthesia with isoflurane and resection of the left hepatic lobe, a microvascular clamp was placed for 60 minutes across the portal triad supplying the median lobe. Congestion of the bowel was prevented during the clamping period through the preservation of the portal flow through the right and caudate lobes. At the end of ischemia, the right lobe and caudate lobes were resected, and reperfusion of the median lobe was achieved by the release of the clamp.

Experimental Design

Protocol 1

The effects of RBP4 and retinol on the parameters of liver regeneration and damage 24 hours after reperfusion were examined:

1. Sham group (Ln and Ob rats). The hepatic hilar vessels of the animals were dissected.
2. PH+I/R group (Ln and Ob rats). The animals underwent PH (70%) with 60 minutes of ischemia.[15]
3. PH+I/R+RBP4 group (Ln and Ob rats). The animals were treated as the animals in group 2 were, but they were also treated with RBP4 (5 μg/kg intravenously) before the surgical procedure.[8]
4. PH+I/R+retinol group (Ln and Ob rats). The animals were treated as the animals in group 2 were, but they were also treated with retinol (10 mg/kg intraperitoneally) before the surgical procedure.[16]
5. PH+I/R+RBP4+retinol group (Ob rats). The animals were treated as the animals in group 2 were, but they were also treated with RBP4 (5 μ/kg intravenously) and retinol (10 mg/kg intraperitoneally) before the surgical procedure.[8,16]

RBP4 and retinol levels, hepatic damage (transaminases and damage scores), liver regeneration parameters [percentages of Ki-67–positive hepatocytes and levels of hepatocyte growth factor (HGF) and transforming growth factor β (TGF-β)], retinyl ester and TTR levels, and RBP4/retinol ratios were determined for the groups corresponding to protocol 1 24 hours after reperfusion.

Protocol 2

This protocol involved the reperfusion time–dependent effects of retinol on hepatic damage, proliferative activity, and the degree of steatosis. To establish a relationship between the effects of retinol and proliferative activity in steatotic and nonsteatotic livers, we subjected animals to interventions similar to those used for groups 2 and 4 (protocol 1), but the samples were obtained 12, 24, and 48 hours after reperfusion.

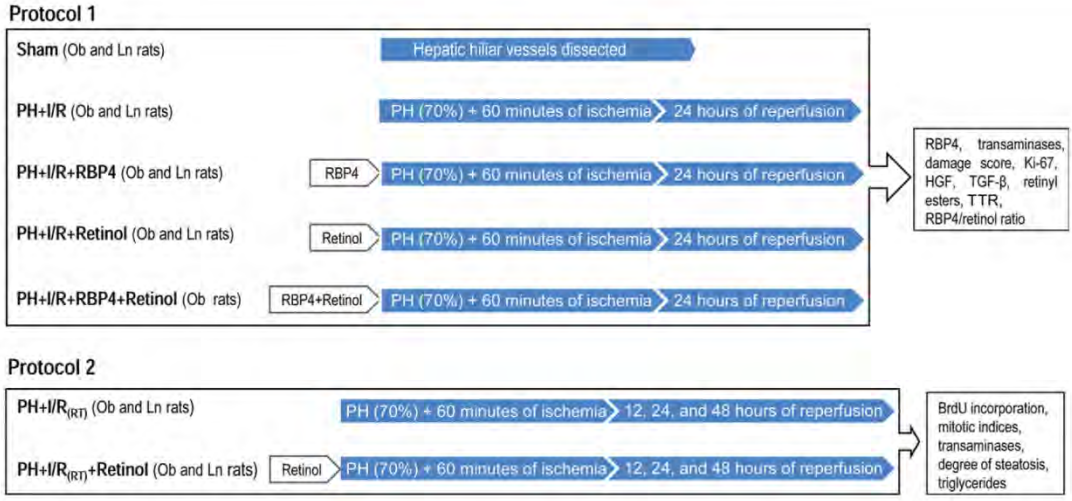

Figure 1. Flow chart of the interventions and measurements corresponding to protocols 1 and 2: (1) effects of RBP4 and retinol on the parameters of liver regeneration and damage 24 hours after reperfusion in the PH+I/R, PH+I/R+RBP4, PH+I/R+retinol, and PH+I/R+RBP4+retinol groups and (2) reperfusion time–dependent effects of retinol on hepatic damage, proliferative activity, and the degree of steatosis throughout reperfusion (12-48 hours) in the PH+I/$R_{(RT)}$ and PH+I/$R_{(RT)}$+retinol groups.

Bromodeoxyuridine (BrdU) was administered intraperitoneally at 50 mg/kg 1 hour before the animals were sacrificed at the indicated times, and they were processed to determine the incorporation of BrdU and mitotic indices.[17] Under these conditions, hepatic damage (transaminases and damage scores) and the degree of steatosis were also evaluated.

The interventions and measurements used in protocols 1 and 2 are shown in Fig. 1. The doses and pretreatment times used for RBP4 and retinol were selected on the basis of previous studies[8,16] and preliminary studies by our group. Control experiments were performed with the corresponding vehicle for each drug (saline and dimethyl sulfoxide for RBP4 and retinol, respectively).

Biochemical Determinations

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), HGF (a potent mitogen), total and active TGF-β (considered the main inhibitor of hepatic proliferation), RBP4, and triglycerides were measured as described elsewhere.[8,15,18]

Analytical Determination of Retinol and Retinyl Esters

Retinol and retinyl esters were measured with a reversed-phase high-performance chromatography system (Waters, Eschborn, Germany).[19] Vitamin A was extracted from the plasma and liver and was separated on a C18 column (Repro-Sil 70, Alltech Grom, Rottenburg-Hailfingen, Germany). Retinol and retinyl esters were quantified by the measurement of the absorption at 325 nm with a photodiode array detector (model 996, Waters). The detection limits for retinol and retinyl palmitate were 2.0 and 2.4 ng, respectively; the coefficient of variation between runs was 4%, and the recovery rate was greater than 95%.

Real-Time Reverse-Transcription Polymerase Chain Reaction

Quantitative real-time reverse-transcription polymerase chain reaction analyses were performed with Assays-on-Demand TaqMan probes (Rn01451317_g1 for RBP4 and Rn00667869_m1 for β-actin, Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.[8]

Western Blotting

Western blotting for TTR was performed as described elsewhere.[20] Anti-TTR antibodies were acquired from DakoCytomation (Hamburg, Germany). Immunoreactive protein bands were visualized with chemiluminescence reagents and were quantified densitometrically with Quantity One software.

Histology, Red Oil Staining, and Immunohistochemistry

To appraise the severity of hepatic injury, we graded hematoxylin and eosin–stained sections with a point-counting method on an ordinal scale: (0) minimal or no evidence of injury; (1) mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; (2) moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and a loss of intercellular borders; (3) severe necrosis with

disintegration of hepatic cords, hemorrhaging, and neutrophil infiltration; and (4) very severe necrosis with disintegration of hepatic cords, hemorrhaging, and neutrophil infiltration.[8,21] Liver steatosis was evaluated via red oil staining on frozen specimens, and the percentage of steatosis was calculated by image analysis according to the standard procedure.[18] For liver regeneration, liver samples were immunostained with a rabbit monoclonal antibody against Ki-67 (clone SP6, Abcam, Cambridge, MA), developed with diaminobenzidine, and counterstained with hematoxylin.[22] The percentages of proliferating hepatocytes were also estimated through the quantification of hepatocytes that incorporated BrdU. BrdU-positive cells were detected with a mouse anti-BrdU antibody (GE Healthcare, United States).[17] The mitotic index was determined in hematoxylin and eosin–stained liver sections.[15] At least 30 high-power fields were counted.

Statistics

Data are expressed as means and standard deviations, and they were compared statistically via a 1-way analysis of variance and then a post hoc Student-Newman-Keuls test; a P value < 0.05 was considered significant. The Spearman correlation coefficient was used to investigate correlations between the effects of retinol therapy and the degree of steatosis; P values < 0.05 were considered statistically significant.

RESULTS

Effect of RBP4 24 Hours After Reperfusion

RBP4 Levels in Steatotic and Nonsteatotic Livers

The RBP4 messenger RNA (mRNA) and protein levels in nonsteatotic and steatotic livers from the PH+I/R group were lower than the levels in livers from the sham group (Fig. 2). The plasma RBP4 levels showed a pattern similar to that described for hepatic RPB4 levels (data not shown).

Hepatic Damage and Regeneration

The administration of RBP4 exacerbated hepatic damage in steatotic and nonsteatotic livers from the PH+I/R+RBP4 group and increased damage scores and transaminase levels in comparison with the PH+I/R group (Fig. 3A). The number of Ki-67–positive hepatocytes in both liver types was lower for the PH+I/R+RBP4 group versus the PH+I/R group. This decrease in proliferative cells was associated with low HGF levels and high levels of active TGF-β (Fig. 3A); the total hepatic TGF-β levels were similar for all groups (data not shown). We confirmed that the administration of RBP4 at the used dose raised RBP4 levels in both liver types with respect to the levels in the sham group. The RBP4 (pmol/g of tissue) protein levels in steatotic livers from the sham, PH+I/R, and PH+I/R+RBP4 groups were 242.17 ± 19.55, 82.41 ± 3.91, and 239.18 ± 20.62, respectively ($P < 0.05$ for the PH+I/R+RBP4 group versus the PH+I/R group; P = not significant for the PH+I/R+RBP4 group versus the sham group).

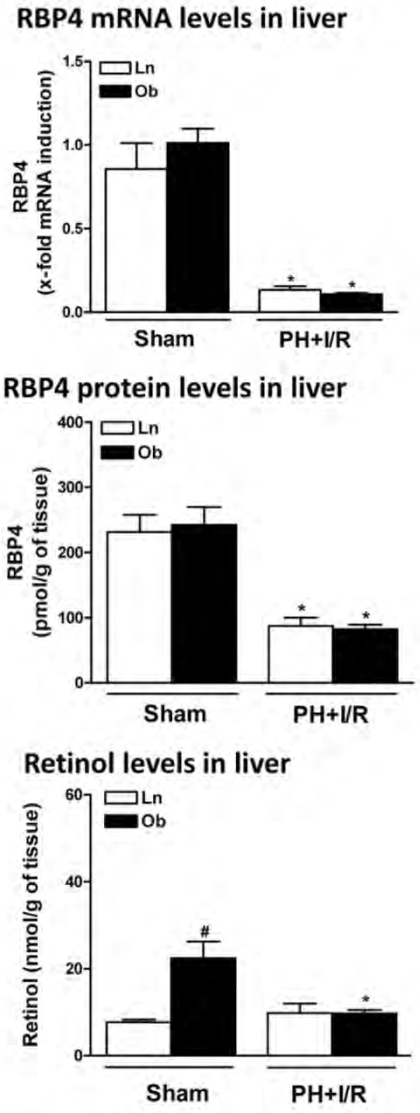

Figure 2. RBP4 mRNA and protein levels and retinol levels in both liver types 24 hours after reperfusion. Six Ln animals and 6 Ob animals from each group were included for each measurement. The retinol levels in steatotic livers from the sham group were higher than the levels in nonsteatotic livers. The polymerase chain reaction fluorescent signals for RBP4 were normalized to the signals obtained from an endogenous reference (β-actin). The β-actin–normalized RBP4 mRNA levels were calculated with respect to the levels of the sham control group with the $2^{-\Delta\Delta C_t}$ method. $^{*}P < 0.05$ versus sham animals; $^{\#}P < 0.05$.

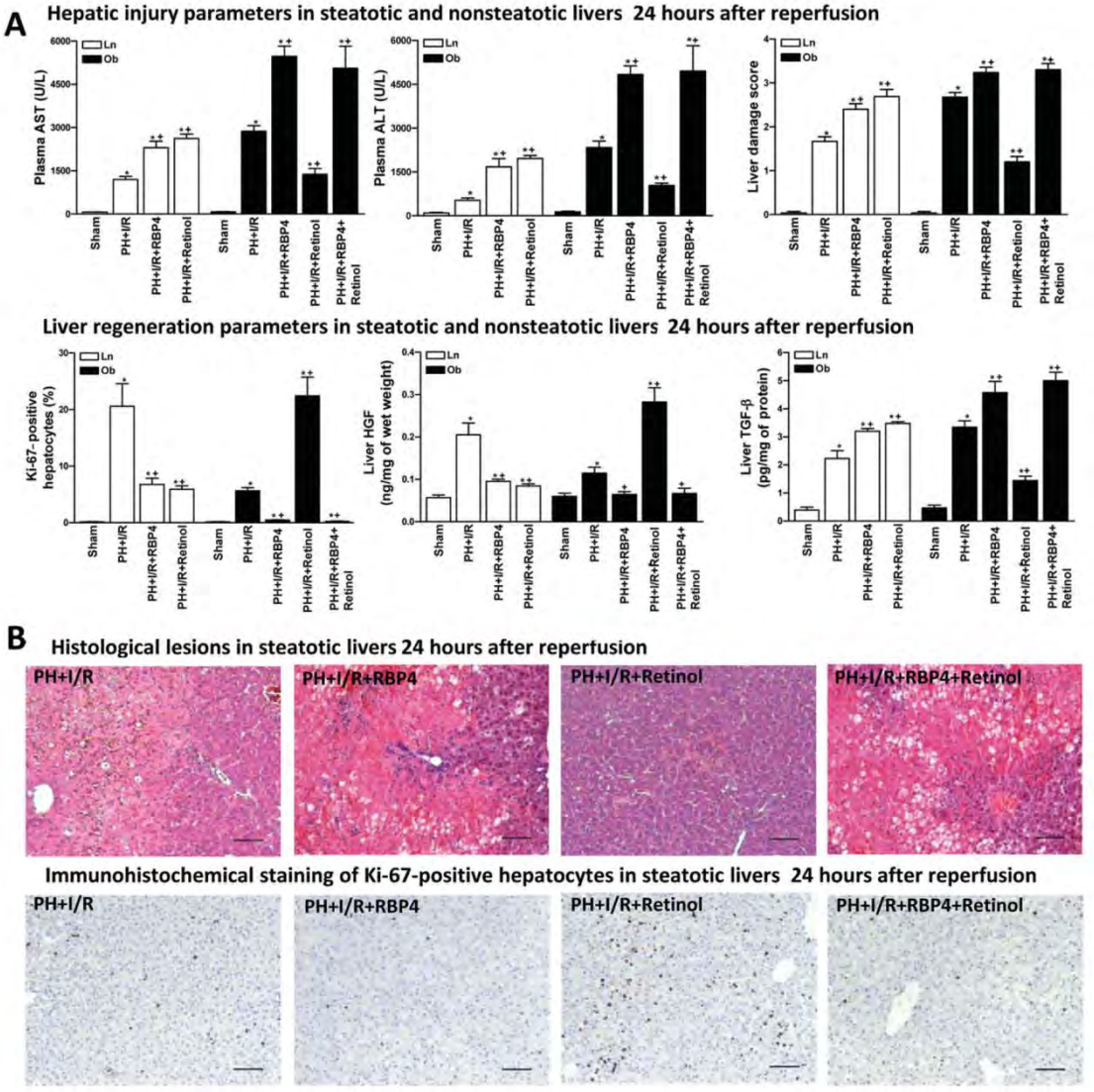

Figure 3. (A) Effects of RBP4 and retinol (separately or in combination) on hepatic injury (plasma AST and ALT levels and liver damage scores) and liver regeneration parameters (percentages of Ki-67–positive hepatocytes and plasma HGF and TGF-β levels) 24 hours after reperfusion. Six Ln animals and 6 Ob animals from each group were included for each measurement. $^{*}P < 0.05$ versus sham animals; $^{+}P < 0.05$ versus PH+I/R animals. (B) Representative photographs of hematoxylin and eosin staining 24 hours after reperfusion show extensive areas of coagulative necrosis in the PH+I/R, PH+I/R+RBP4, and PH+I/R+RBP4+retinol groups and small areas of coagulative necrosis in the retinol group (scale bar = 1000 μm). Representative photographs of immunohistochemical staining of Ki-67–positive hepatocytes in steatotic livers 24 hours after reperfusion show that the number of Ki-67–positive hepatocytes was lower in the PH+I/R, RBP4, and RBP4+retinol groups versus the retinol group (scale bar = 1000 μm).

Effect of Retinol 24 Hours After Reperfusion

Retinol Levels in Steatotic and Nonsteatotic Livers

In nonsteatotic livers, the retinol levels for the PH+I/R group were similar to those for the sham group. The retinol levels in steatotic livers from the sham group were significantly higher than the levels in nonsteatotic livers (Fig. 2), and this indicated that the presence of fatty infiltration in and of itself (without any surgical intervention) induced changes in retinol metabolism, as previously suggested.[23] The retinol

levels in steatotic livers from the PH+I/R group were reduced in comparison with the sham group.

Hepatic Damage and Regeneration

The effects of retinol were dependent on the type of liver. The administration of retinol negatively affected hepatic damage and liver regeneration in nonsteatotic livers from the PH+I/R+retinol group because the parameters of hepatic injury were higher than those for the PH+I/R group. This was associated with reduced percentages of Ki-67–positive hepatocytes, reduced HGF levels, and high TGF-β levels in comparison with the PH+I/R group (Fig. 3A). The administration of retinol to Ob animals in the PH+I/R+retinol group reduced hepatic damage; this was indicated by the reduction in transaminases and damage scores in comparison with the PH+I/R group. Retinol administration also increased the percentage of Ki-67–positive hepatocytes in steatotic livers in comparison with the PH+I/R group. This improvement was associated with high HGF levels and low TGF-β levels (Fig. 3A).

Effects of RBP4 and Retinol in Combination 24 Hours After Reperfusion

Taken together, the results presented up to this point show that neither the administration of RBP4 nor the administration of retinol protects nonsteatotic livers in the setting of PH under I/R. Because of our demonstration of the differential effects of RBP4 and retinol on steatotic livers and data indicating that changes in RBP4 induce changes in retinol levels,[10,11] we evaluated whether the injurious effects of RBP4 with respect to damage and regeneration in steatotic livers could be explained by changes in retinol levels.

Hepatic Damage and Regeneration

The combined administration of RBP4 and retinol resulted in injury and regeneration parameters for steatotic livers from the PH+I/R+RBP4+retinol group that were similar to those for steatotic livers from the PH+I/R+RBP4 group (Figs. 3A). The histological analysis revealed severe, extensive, and confluent areas of coagulative necrosis with neutrophil infiltration in steatotic livers from the PH+I/R, PH+I/R+RBP4, and PH+I/R+RBP4+retinol groups (Fig. 3B). The liver damage scores for the PH+I/R+RBP4 and PH+I/R+RBP4+retinol groups were significantly higher than those recorded for the PH+I/R group. Both the number and extent of necrotic areas in steatotic livers were reduced by retinol. In comparison with the PH+I/R group, the PH+I/R+retinol group also exhibited an increased number of Ki-67–positive hepatocytes in steatotic livers. The number of Ki-67–positive hepatocytes in the PH+I/R+RBP4 and PH+I/R+RBP4+retinol groups was lower than the number in the PH+I/R group (Fig. 3A,B). Thus, the administration of RBP4, separately or in combination with retinol, negatively affected hepatic damage and regeneration.

Retinyl Esters and Retinol in Liver and Plasma

Vitamin A is stored in the liver as retinyl esters, and they must be hydrolyzed into retinol before vitamin A can be mobilized into the circulation.[4] In line with this, retinyl ester and retinol levels in steatotic livers were reduced for the PH+I/R group versus the sham group (Fig. 4A). This decrease was associated with high plasma retinol levels (Fig. 4B). As previously suggested on the basis of studies in cultured cells,[24] RBP4 could affect the storage and mobilization of retinol in steatotic livers. In fact, we found that retinyl ester levels (but not retinol levels) were increased in steatotic livers (Fig. 4A) and circulating retinol levels were reduced (Fig. 4B) for the PH+I/R+RBP4 group versus the PH+I/R group or the sham group. As expected, retinol administration increased both retinyl ester and retinol levels in steatotic livers from the PH+I/R+retinol and PH+I/R+RBP4+retinol groups versus the PH+I/R group and increased retinyl ester levels (but not retinol levels) in plasma (Fig. 4A,B). Our results confirmed that RBP4 administration led to higher RBP4 levels in steatotic livers from the PH+I/R+RBP4 and PH+I/R+RBP4+retinol groups versus the PH+I/R group. For instance, the hepatic RBP4 levels for the PH+I/R+RBP4+retinol and PH+I/R groups were 239.6 ± 21.65 and 82.41 ± 3.91 respectively ($P < 0.05$). The hepatic RBP4 (pmol/g of tissue) levels for the PH+I/R+retinol group (83.45 ± 4.56) were similar to the levels for the PH+I/R group (82.41 ± 3.91, P = not significant).

Transport of Retinol in Plasma

Reduced plasma RBP4 levels were observed for the PH+I/R group versus the sham group (Fig. 4C). The plasma RBP4 levels for the PH+I/R+RBP4 and PH+I/R+RBP4+retinol groups were lower than those for the PH+I/R group. The plasma RBP4 levels for the PH+I/R+retinol group were similar to those for the PH+I/R group. The plasma TTR levels were reduced for the PH+I/R group versus the sham group, whereas the TTR levels for the PH+I/R+RBP4, PH+I/R+retinol, and PH+I/R+RBP4+retinol groups were similar to those for the sham group. It has been reported that retinol is normally found in plasma in a 1:1 molar ratio with RBP4.[25] In the sham group, the RBP4/retinol ratio was approximately 2; in the PH+I/R and PH+I/R+RBP4 groups, the RBP4/retinol ratio was greater than 1; and in the PH+I/R+retinol and PH+I/R+RBP4+retinol groups, the RBP4/retinol ratio was less than 1 (Fig. 4C).

Reperfusion Time–Dependent Effect of Retinol on Hepatic Damage, Proliferative Activity, and Degree of Steatosis

Hepatic damage and proliferative activity in steatotic and nonsteatotic livers were measured throughout reperfusion (12, 24, and 48 hours; Fig. 5). In Ln animals, the parameters of hepatic injury were higher for the partial hepatectomy under ischemia-reperfusion

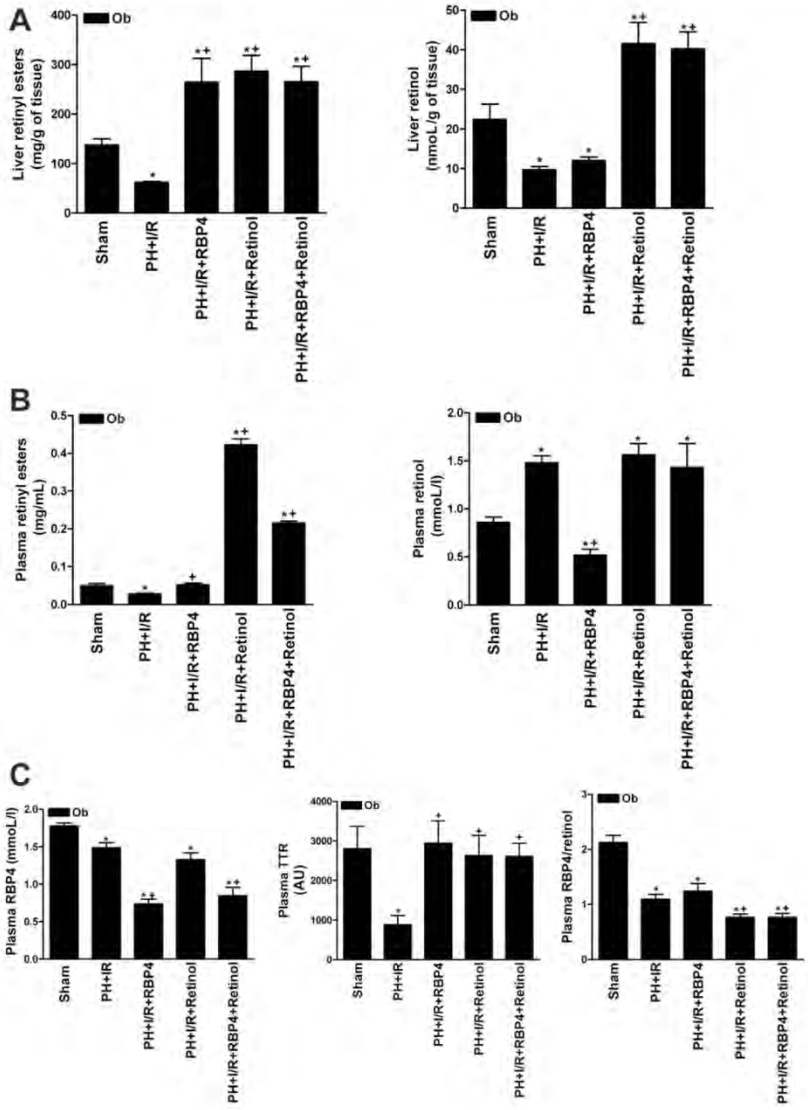

Figure 4. Effects of RBP4 and retinol (separately or in combination) on retinyl esters and retinol in (A) steatotic livers and (B) plasma and (C) RBP4 and TTR levels and RBP4/retinol ratios in plasma 24 hours after reperfusion. Six Ob animals from each group were included for each measurement. $^{*}P < 0.05$ versus sham animals; $^{+}P < 0.05$ versus PH+I/R animals.

(PH+I/R) at different reperfusion times (RT) treated with retinol (PH+I/$R_{(RT)}$+retinol) group versus the partial hepatectomy under ischemia-reperfusion (PH+I/R) at different reperfusion times (RT) (PH+I/$R_{(RT)}$) group at each time point (Fig. 5A). In Ln animals, the administration of retinol impaired the mitotic index and hepatocellular BrdU incorporation after reperfusion. For instance, the percentages for BrdU-positive hepatocytes were 9.3% ± 1.09% and 19.5% ± 1.67% in the PH+I/$R_{(RT)}$+retinol and PH+I/$R_{(RT)}$ groups, respectively, 48 hours after reperfusion. In Ob animals, the hepatic damage parameters were lower for the PH+I/$R_{(RT)}$+retinol group versus the PH+I/$R_{(RT)}$ group at each time (Fig. 5B). Our results revealed an improvement of the regenerative process in steatotic livers from the PH+I/$R_{(RT)}$+retinol group versus the PH+I/$R_{(RT)}$ group. Thus, 48 hours after reperfusion, the percentage of BrdU-positive hepatocytes was 17.3% ± 0.99% for the PH+I/$R_{(RT)}$+retinol group, whereas only 6.5% ± 0.76% of BrdU-labeled hepatocytes were detected at that reperfusion time in the PH+I/$R_{(RT)}$ group (Fig. 5B). Our results suggest a relationship between the effects of retinol therapy and the degree of steatosis in steatotic livers. Forty-eight hours after reperfusion, increased hepatocellular fatty infiltration and triglyceride levels were observed in the PH+I/$R_{(RT)}$ group versus the sham group (Fig. 5C,D). This is in line with a previous report of hepatic lipid

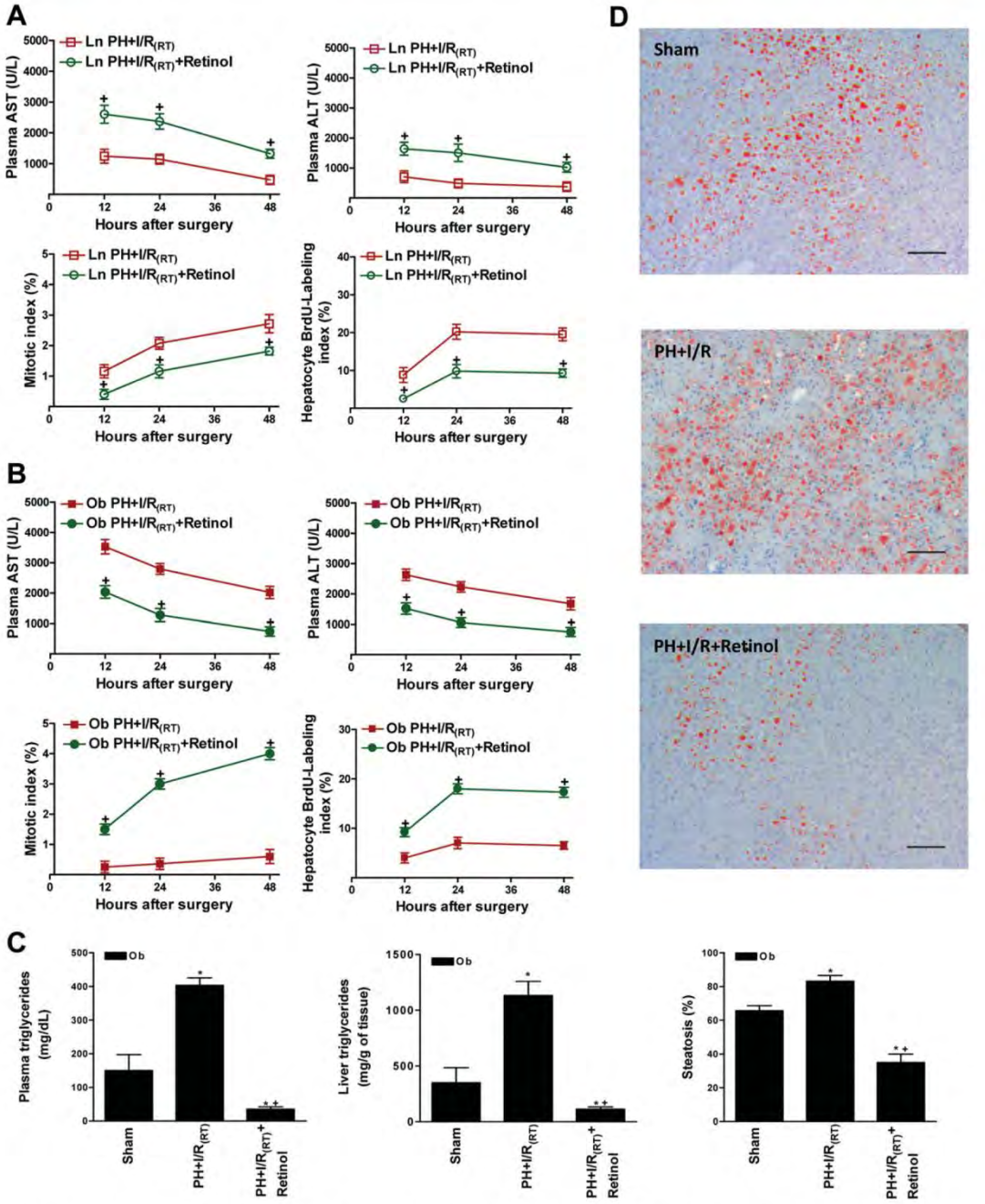

Figure 5. Effects of retinol on hepatic injury (plasma AST and ALT levels) and liver regeneration (percentages of BrdU-positive hepatocytes and mitotic indices) throughout reperfusion (12, 24, and 48 hours) in (A) Ln animals and (B) Ob animals. Eighteen Ln animals and 18 Ob animals (6 Ln and 6 Ob animals for each reperfusion time) were included for each measurement. (C) Triglyceride levels and steatosis percentages and (D) representative photographs of red oil staining 48 hours after reperfusion are shown. Steatotic livers from the retinol group showed reduced fat globules (red) in hepatocytes in comparison with steatotic livers from the sham and PH+I/R groups (scale bar = 1000 μm). Six Ob animals from each group were included for each measurement. *$P < 0.05$ versus sham animals; $^{+}P < 0.05$ versus PH+I/R animals.

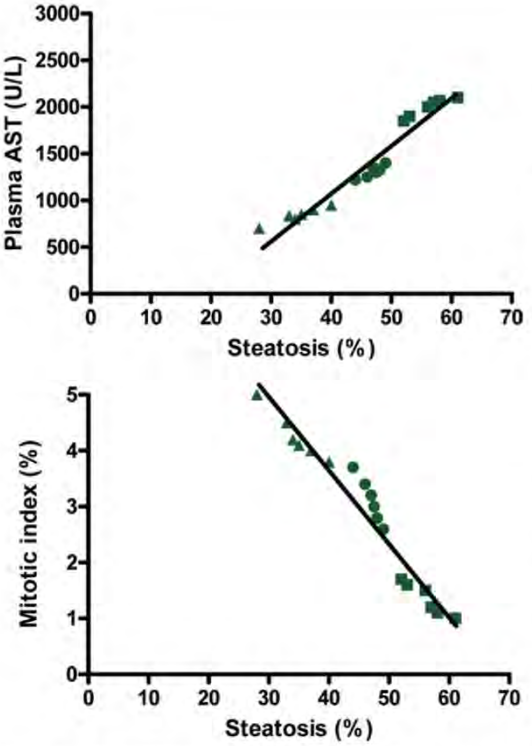

Figure 6. Relationship between the degree of steatosis and (A) the plasma AST level ($r = 0.9677$, $P < 0.05$) and (B) the mitotic index ($r = -0.9724$, $P < 0.05$): (■) Ob PH+I/$R_{(IRT)}$+retinol animals 12 hours after reperfusion, (●) Ob PH+I/$R_{(IRT)}$+retinol animals 24 hours after reperfusion, and (▲) Ob PH+I/$R_{(IRT)}$+retinol animals 48 hours after reperfusion.

accumulation during regeneration after PH.[26] The treatment with retinol decreased the accumulation of fatty droplets and triglycerides in steatotic livers from the PH+I/$R_{(IRT)}$+retinol group versus the PH+I/$R_{(IRT)}$ group. The steatosis percentages were 35.0% ± 1.78% and 83.13% ± 3.40% for the PH+I/$R_{(IRT)}$+retinol and PH+I/$R_{(IRT)}$ groups, respectively, 48 hours after reperfusion. Figure 6 shows the relationship between the effects of retinol therapy and the degree of steatosis 12, 24, and 48 hours after reperfusion. A significant correlation between the degree of fat infiltration and the plasma AST level ($r = 0.9677$, $P < 0.05$) and an inverse correlation between the degree of fat infiltration and the mitotic index ($r = -0.9724$, $P < 0.05$) were observed. Treatment with retinol, therefore, attenuated hepatic damage and improved hepatocellular proliferation during liver regeneration. This was associated with a reduced accumulation of hepatocellular fat.

DISCUSSION

The decreased RBP4 levels observed in the circulation after PH under I/R might reflect decreased hepatic synthesis of RBP4 due to a loss of functional hepatic tissue. Because the liver is the main site of RBP4 synthesis,[8,27] hepatocyte damage decreases circulating RBP4 levels. Moreover, the synthesis of negative acute-phase proteins such as RBP4 might be reduced in the liver during inflammation or hepatic resection as part of a compensatory response to the sudden increase in the synthesis of positive acute-phase proteins.[11] The effects of RBP4 could depend on the surgical conditions. Indeed, in contrast to steatotic liver transplantation,[8] RBP4 administration worsens liver damage and regenerative failure in the setting of PH under I/R.

Retinol is decreased in steatotic livers after PH under I/R and is increased in plasma. This is consistent with the increased secretion of retinol from steatotic livers into the circulation. However, other hypotheses proposed for different liver diseases,[28] including increased catabolism of retinol in the liver and decreased liver uptake of retinyl esters formed in peripheral tissues, should not be discounted. Retinol is normally found in plasma in a 1:1 molar ratio with RBP4 (the RBP4/retinol ratio is approximately 1.0).[29] However, in our study and in agreement with the reports of others,[4] the RBP4/retinol ratio for the sham group of Ob (steatotic) animals was approximately 2, and this indicated that the proportion of RBP4 in plasma was higher than the proportion of retinol. In this context, it should be considered that RBP4 is present as a holoform (retinol-bound) and an apoform (retinol-unbound) because RBP4 may transport and deliver other lipophilic molecules in addition to retinol.[6,30] A question that arises is how the high plasma retinol levels in the PH+I/R group are maintained in light of the low plasma RBP4 and TTR levels. Like RBP4, TTR is a negative acute-phase protein, and the hepatic syntheses of both are also decreased during inflammation.[11] However, because the plasma RBP4/retinol ratio in the PH+I/R group was greater than 1, there was enough RBP4 to bind retinol despite the reduced RBP4 levels. It could be assumed that the reduced plasma TTR levels in the PH+I/R group were also high enough to maintain the retinol-RBP4 complex in plasma, insofar as there is usually a 3- to 5-fold molar excess of circulating TTR with respect to circulating RBP4.[11,27] The lack of a correlation between RBP4 and TTR levels in plasma under our surgical conditions could be explained, at least in part, by the differential clearance of RBP4 and TTR by the kidneys: TTR has a higher molecular weight and is, therefore, retained to a greater extent than RBP4.[27] Moreover, TTR has additional functions (eg, the transporter for T4).[4]

RBP4 exerted injurious effects on steatotic livers with respect to damage and regeneration independently of retinol. Moreover, the changes in retinol metabolism induced by RBP4 could have occurred simply as a result of disease progression and thus do not explain the injurious effects of RBP4. The mechanism behind this is unclear. Retinyl esters seem to be affected by RBP4 administration, and this also

suggests that RBP4 has actions independent of simple retinol transport. However, the RBP4-induced increase in retinyl esters does not explain the injurious effects of RBP4. Indeed, the loss of retinoid (rather than an increase in retinoid levels) contributes to the development of hepatic diseases.[31]

Serum RBP4 levels are elevated in insulin-resistant mice and humans with obesity and type 2 diabetes. The transgenic overexpression of human RBP4 or the injection of recombinant RBP4 into normal mice causes insulin resistance. Conversely, the genetic deletion of RBP4 enhances insulin sensitivity.[6] Elucidating whether RBP4 also has metabolic effects was not an aim of the present study. Nevertheless, because of our results, this seems to not be the case for our experimental model. The Ob Zucker rats used in the present study have a mutated leptin receptor and, as a result, are hyperphagic, Ob, and hyperinsulinemic. They are insulin-resistant, but they have normal blood glucose levels. Ob Zucker rats do not develop diabetes. Ln Zucker rats maintain an Ln phenotype throughout life, and they show normal blood insulin and glucose levels. RBP4 administration did not alter the plasma insulin or glucose levels in the Ob Zucker rats undergoing PH and I/R (data not shown). Under our conditions, there was no relationship between RBP4 levels and insulin resistance. Indeed, Ln and Ob Zucker rats in the sham group showed similar RBP4 levels in the liver and plasma, and only Ob Zucker rats are insulin-resistant. In contrast, Yang et al.[6] have shown that genetically Ob mice and high-fat diet–induced Ob mice with insulin resistance exhibit increased plasma RBP4 in comparison with Ln controls. The differences in plasma RBP4 levels observed in the 2 studies can be explained at least partially by the differences in RBP4 regulation between rats and mice.[32] All these data indicate that under our conditions, RBP4 does not affect insulin resistance.

Further studies, which are not part of the objectives of the present study, are required to answer why the pharmacological modulation of RBP4 exerted damaging effects in the setting of PH under I/R. It is possible that the compensatory changes in the protein synthesis of positive and negative acute-phase proteins, which were necessary to restore protein homeostasis after hepatic resection, were disturbed in the remaining liver when RBP4 was administered. From a clinical perspective, strategies based on modulating RBP4 might not be appropriate for hepatic resection or under surgical conditions (including small-for-size liver transplantation). When we administered RBP4 to compensate for the reduced RBP4 levels induced by liver surgery, we observed more injurious effects with respect to damage and liver regeneration.

In contrast, pretreatment with retinol alone may create new possibilities for therapeutic interventions in the resection of steatotic livers. As expected, retinol administration alone increased vitamin A storage in the liver. The increase in retinyl esters (but not retinol or RBP4) in plasma after retinol administration suggests that retinol is incorporated as retinyl esters in hepatocytes and is secreted together with lipoproteins. Thus, retinol administration could supply vitamin A to target tissues. Data indicate that the RBP4 molecule contains 1 binding site for a single molecule of retinol,[25] so the fact that the RBP4/retinol ratio after retinol administration was less than 1 might suggest that a fraction of retinol is bound to carriers other than RBP4, just as with other pathologies.[33]

The results presented here indicate that retinol reduces damage and improves liver regeneration in an experimental model combining PH and I/R. Unpublished results from our group indicate that retinol therapy for steatotic livers affects both I/R injury and PH. Thus, retinol reduces hepatic damage in Ob rats subjected to partial hepatic ischemia (60 minutes) without hepatectomy. Retinol administration also reduces hepatic damage and improves liver regeneration in Ob rats subjected to 70% resection only (data not shown).

Further studies will be required to explain how retinol reduces lipid accumulation in the regenerating liver after surgery. The effects of retinol on the mobilization of fatty acids from peripheral stores and hepatic lipogenesis, fatty acid oxidation, and triglyceride secretory mechanisms in the liver should be explored as possible answers to this question. Our results show that the benefits of retinol with respect to damage and liver regeneration in steatotic livers are associated with reduced hepatic lipid accumulation. It has been reported that it is crucial to reduce steatosis to prevent the vulnerability of steatotic livers to I/R injury and regenerative failure.[34]

In conclusion, PH under I/R affects hepatic vitamin A metabolism by reducing hepatic RBP4 expression and increasing the mobilization of retinol into the circulation. The results presented here suggest that under surgical conditions requiring liver regeneration, modulating RBP4 levels worsens the outcome and is, therefore, not advised. This study also points to new possibilities for therapeutic interventions based on retinol pretreatment to protect steatotic livers against damage and regenerative failure after liver surgery.

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THE BENEFICIAL EFFECTS OF RETINOL IN STEATOTIC LIVERS UNDERGOING LIVER SURGERY ARE MEDIATED BY PPARγ

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INTRODUCTION

Ischemia-reperfusion (I/R)-which is commonly applied in clinical practice to reduce bleeding, increase hepatic damage and impairs regeneration in steatotic livers.[1,2] Since retinol levels in steatotic livers decrease significantly after partial hepatectomy under ischemia-reperfusion,[3] we examined whether retinol administration could protect steatotic and non-steatotic livers in partial hepatectomy under I/R. The possibility that retinol performs its functions in steatotic livers by reducing oxidative stress and/or regulating certain transcription factors, including PPARs has been previously suggested.[4,5,6] Thus, we studied retinol mechanisms of action in our model.

EXPERIMENTAL GROUPS

Male, lean (Ln) and obese (Ob) rats of 14-16 weeks old were used in these experiments. Six Ln animals and six Ob animals were used in each group.
This study complied with European Union regulations on animal experiments (directive 86/609/EEC).

1. Sham
Ln and Ob Zucker rats. Hepatic hiliar vessels were dissected.
→ Hiliar vessels dissection

2. PH+I/R
The animals underwent PH (70%) with 60 minutes of ischemia.
→ Partial hepatectomy → 24 h reperfusion

3. PH+I/R+RBP4
Same as group 2, but they were also treated with RBP4 (5 µg/kg intravenously) before the surgical procedure
RBP4 → Partial hepatectomy → 24 h reperfusion

4. PH+I/R+Retinol
Same as group 2, but they were also treated with Retinol (10 µg/kg intravenously) before the surgical procedure
Retinol → Partial hepatectomy → 24 h reperfusion

5. PH+I/R+Retinol
Same as group 2, but they were also treated with Retinol (10 µg/kg intravenously) and the PPARγ-antagonist GW9662 (1000µg/kg) before the surgical procedure
Retinol + GW9662 → Partial hepatectomy → 24 h reperfusion

RESULTS

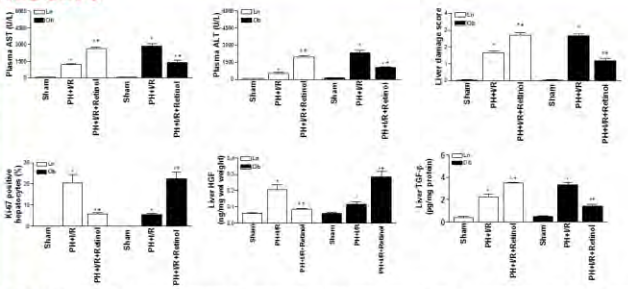

Figure 1. *Effects of retinol administration on hepatic injury and liver regeneration.* Retinol administration negatively affected hepatic damage and liver regeneration in nonsteatotic livers from PH+I/R+retinol group. Retinol administration to Ob rats reduced liver injury and decreased transaminase levels and damage score in comparison with PH+I/R group. Retinol pre-treatment negatively affected liver regeneration in nonsteatotic livers from PH+I/R+retinol group. The administration of retinol increased the percentage of ki-67-positive hepatocytes, which was associated with increased levels of HGF and low levels of active TGF-β. *P < 0.05 versus Sham; +P < 0.05 versus PH+I/R.

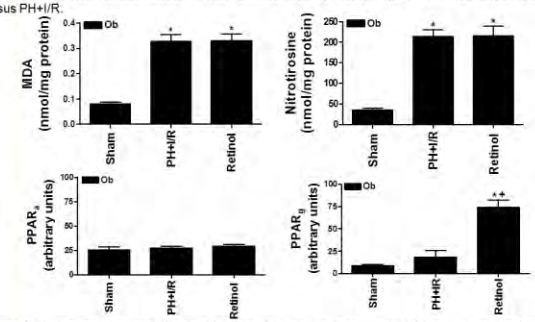

Figure 2. *Action mechanisms of retinol in steatotic livers undergoing partial hepatectomy under I/R.* Oxidative stress and PPAR levels in steatotic livers. Retinol administration resulted in MDA and nitrotyrosine levels similar to those detected in the PH+I/R group, indicating that retinol did not modify oxidative stress in steatotic livers. Retinol did not alter PPARα but did increase PPARγ levels in steatotic livers compared with the PH+I/R group. *P < 0.05 versus Sham; +P < 0.05 versus PH+I/R.

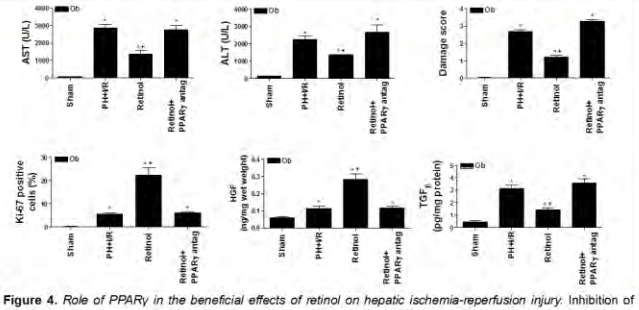

Figure 4. *Role of PPARγ in the beneficial effects of retinol on hepatic ischemia-reperfusion injury.* Inhibition of PPARγ in the presence of retinol (Retinol+PPARγ antag group) abolished the beneficial effects of retinol on damage and regeneration in steatotic livers. Transaminase levels and damage scores in the Retinol+PPARγ antag group were similar to those in the PH+I/R group. The Retinol group also exhibited an increased number of Ki67-positive hepatocytes in steatotic livers compared with the PH+I/R group, indicating enhanced liver regeneration with retinol administration. This was associated with high HGF and low TGFβ levels. Again, the percentage of Ki67-positive hepatocytes and HGF and TGFβ values were similar in the Retinol+PPARγ antag group and the PH+I/R group. Collectively, these results strongly suggest that the beneficial effects of retinol are mediated by PPARγ. *P < 0.05 versus Sham; +P < 0.05 versus PH+I/R.

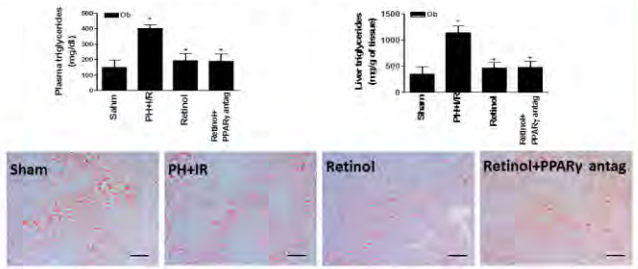

Figure 5. *Effects of retinol administration on lipid accumulation in steatotic livers.* PH+I/R increased plasma and liver triglyceride levels and hepatocellular fatty infiltration compared with the Sham group, whereas retinol reduced hepatic fat accumulation during liver regeneration. Triglycerides levels and hepatocyte fatty infiltration in the Retinol+PPARγ antag group were similar to those in the Retinol group, indicating that the actions of retinol in this context were independent of PPARγ.

CONCLUSION

The effects of retinol were dependent on the type of liver. Retinol administration to compensate for the reduced retinol levels induced by liver surgery exacerbated the negative consequences of liver surgery on damage and liver regeneration in nonsteatotic liver; whereas retinol administration to obese animals had protective effects. In our hands, the administration of retinol protected only steatotic livers, and did so through a mechanism independent of oxidative stress. Our results demonstrate that retinol acts through up-regulation of PPARγ expression to protect steatotic livers against damage and regenerative failure as consequence of liver surgery. In contrast to the key role of PPARγ in these actions of retinol, the effects of retinol on lipid accumulation were PPARγ-independent. Further studies will be required to explain how retinol prevents lipid accumulation in the regenerating liver after surgery. Thus, in terms of clinical applications, this study points to new possibilities for therapeutic interventions based on retinol pretreatment to protect steatotic livers against damage and regenerative failure after liver surgery.

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Potential conflict of interest: Nothing to report

Abstract Preview - Step 3/4

- print version -

Topic: 10a. Fatty Liver Disease: a. Experimental

Title: **PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORγ IS INVOLVED IN THE BENEFIFS OF RETINOL IN STEATOTIC AND NON-STEATOTIC LIVERS UNDERGOING PARTIAL HEPATECTOMY UNDER ISCHEMIA-REPERFUSION**

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Text: Ischemia-reperfusion (I/R)-which is commonly applied in clinical practice to reduce bleeding, increase hepatic damage and impairs regeneration in steatotic livers. We examined whether modulating retinol could protect steatotic and non-steatotic livers in partial hepatectomy under I/R, and if these could be mediated by PPAR. Steatotic and non-steatotic livers from Zucker rats were subjected to partial hepatectomy (70%) under 60 min of ischemia (PH+I/R). Retinol levels were measured and altered pharmacologically and their effects on hepatic damage and regeneration were studied after reperfusion. Retinol levels in steatotic livers from sham group were significantly higher than the levels in non-steatotic livers, indicating that the presence of fatty infiltration in and of itself induce changes in retinol metabolism. Thus, the effects of retinol were dependent on the type of liver. In non-steatotic livers, retinol levels in animals subjected to PH+I/R were similar to those of the Sham group. However, retinol levels in in steatotic livers undergoing PH+I/R were reduced in comparison with the Sham group. The administration of retinol increased hepatic injury and impaired liver regeneration in non-steatotic livers. In steatotic livers, retinol reduced damage and improved regeneration after surgery. These benefits of retinol were associated with reduced hepatocellular fat accumulation. Retinol did not modify oxidative stress or PPARα in steatotic livers, but did increase PPARγ. Retinol actions could be mediated throught PPARγ since inhibition of this latter signaling pathway abolished retinol benefits on regeneration and damage. In contrast to the key role of PPARγ in these actions of retinol, the effects of retinol on lipid accumulation were PPARγ-independent. Thus, in terms of clinical applications, retinol pre-treatment might open new avenues for liver surgery that specifically benefit the steatotic liver.

Author Keywords: Ischemia-Reperfusion, Steatosis, Partial Hepatectomy, Retinol, PPARγ

Preferred Presentation Type: **Poster Presentation**

Conference: The International Liver Congress™ 2013, 48th annual meeting of the European Association for the Study of the Liver (EASL) · Abstract: A-533-0025-00182 · **Status: Submitted (checked)**

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Dear Maria Elias-Miró,

On behalf of the Scientific Programme Committee, we are very pleased to inform you that your abstract A-533-0025-00182 entitled: **"PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORγ IS INVOLVED IN THE BENEFIFS OF RETINOL IN STEATOTIC AND NON-STEATOTIC LIVERS UNDERGOING PARTIAL HEPATECTOMY UNDER ISCHEMIA-REPERFUSION"** has been accepted for both **ePOSTER and PAPER POSTER PRESENTATION** at the International Liver CongressTM 2013 (ILC) to be held in Amsterdam, The Netherlands, April 24-28, 2013.

In order to maintain the high scientific profile of the EASL congress and to remain fair to abstract submitters, all abstracts were anonymously reviewed by 4 independent reviewers.

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LIVER TRANSPLANTATION 17:993-1004, 2011

REVIEW

Does Adiponectin Benefit Steatotic Liver Transplantation?

Maria Elias-Miro,[1] **Marta Massip-Salcedo,**[1,2] **Monica Jimenez-Castro,**[1] **and Carmen Peralta**[1,2]
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Strategies for improving the viability of steatotic donor livers could increase the number of organs suitable for transplantation. There is evidence that adiponectin, the most abundant adipose-specific adipokine, acts as an anti-obesity and anti-inflammatory hormone. Here we review the signaling pathways of adiponectin and the possible therapies based on adiponectin regulation that have been examined or applied clinically. Recent studies on the role of adiponectin in steatotic livers subjected to ischemia/reperfusion are discussed. The data suggest that further investigations are required to determine whether adiponectin is a potential therapeutic target in liver transplantation. ***Liver Transpl 17:993-1004, 2011.*** © **2011 AASLD.**

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The potential use of steatotic livers, which are among the most common types of organs from marginal donors, has become a major focus of investigation for transplantation. However, steatotic livers are more susceptible to ischemia/reperfusion (I/R) injury, and when they are transplanted, they lead to poorer outcomes in comparison with nonsteatotic livers. Indeed, the use of steatotic livers for transplantation is associated with an increased risk of primary nonfunction or dysfunction after surgery.[1-5] Despite advances aimed at reducing hepatic I/R injury (which have been summarized in earlier reviews[1,2]), the results have been inconclusive. Here we review the signaling pathway of adiponectin in steatosis and inflammation, which are key factors in steatotic liver transplantation.[1-5] Recent findings concerning the role of adiponectin in hepatic I/R are also discussed. Our review of pharmacological strategies for regulating adiponectin in patients with liver disease may help researchers to find effective pharmacological strategies for the treatment of hepatic I/R. The potential clinical application of adiponectin in the setting of steatotic liver transplantation is also discussed.

CHARACTERISTICS OF ADIPONECTIN IN HEPATIC I/R

We first outline the main characteristics of adiponectin and adiponectin sources and discuss whether adiponectin could be a predictive factor in clinical liver surgery. A unique feature of the adiponectin structure

Abbreviations: ACC, acetyl coenzyme A carboxylase; AdipoQ, adiponectin, C1Q and collagen domain containing; AMPK, adenosine monophosphate–activated protein kinase; Ang, angiotensin; APPL1, adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1; ATP, adenosine triphosphate; ER, endoplasmic reticulum; ET, endothelin; FAS, fatty acid synthase; FFA, free fatty acid; GPAT, glycerol-3-phosphate acyltransferase; GSH, glutathione; HMW, high molecular weight; HO-1, heme oxygenase 1; IFN, interferon; IL, interleukin; I/R, ischemia/reperfusion; JNK, Jun N-terminal kinase; mRNA, messenger RNA; MyD88, myeloid differentiation factor 88; NO, nitric oxide; PC, ischemic preconditioning; PPAR, peroxisome proliferator-activated receptor; RBP4, retinol-binding protein 4; ROS, reactive oxygen species; siRNA, silent small interfering RNA; SOD, superoxide dismutase; SREBP1, sterol regulatory element-binding protein 1; TLR4, toll-like receptor 4; TNF, tumor necrosis factor; TRAF6, tumor necrosis factor receptor–associated factor 6; TRIF, toll/interleukin-1 receptor domain-containing adaptor inducing interferon-β; UCP2, mitochondrial uncoupling protein 2; UPR, unfolded protein response; XDH, xanthine dehydrogenase; XOD, xanthine oxidase.

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is its ability to assemble into several characteristic isoforms, which include a trimeric complex with a low molecular weight, a hexameric complex with a middle molecular weight, and an oligomeric complex with a high molecular weight (HMW) and 18 or more protomers.[6,7] All 3 complexes are present in the circulation, but the metabolic activities are primarily related to the HMW form,[6,8,9] which is the most active form in the liver. Indeed, observations during warm hepatic ischemia indicate that the predominant isoform in steatotic livers is the HMW isoform.[10]

In the last decade, adipose tissue has emerged as an endocrine organ with a key role in energy homeostasis because its metabolic products, adipokines, exert local, peripheral, and central effects. To date, adipose tissue has been considered the major site for endogenous adiponectin production, although there are other potential sources, including the liver.[10-13] There are 2 hypotheses for the detection of adiponectin in the liver. First, adiponectin may be taken from the circulation by cells.[13] Second, the liver may generate adiponectin.[10,14,15] To the best of our knowledge, only 3 studies have reported changes in adiponectin levels due to hepatic I/R,[10,16,17] and only 1 study[10] has evaluated a potential source of adiponectin. In that study, the data for steatotic livers subjected to warm hepatic ischemia support the second hypothesis because the level of adiponectin messenger RNA (mRNA) expression was higher in steatotic livers versus nonsteatotic livers.[10] No correlations were observed between the adiponectin levels in the circulation and those in the liver.[10] In addition, an increased level of adiponectin mRNA expression was observed in steatotic livers in an isolated perfused liver model, and this suggests that steatotic livers alone can generate adiponectin as a result of I/R.[10] Further studies are necessary to elucidate the role of adipose tissue in the changes in adiponectin levels in steatotic livers subjected to I/R during different surgical procedures. The hepatic and adipose expression of adiponectin receptors 1 and 2 has been measured for liver diseases with conflicting results.[15,18-21] None of the 3 studies of hepatic I/R[10,16,17] have reported the involvement of adiponectin receptors in any mechanisms of adiponectin. Investigations of the structure of the adiponectin receptors may be useful for the design of certain strategies, such as those involving adiponectin receptor antagonists or agonists; they could be useful in elucidating the action mechanisms of adiponectin in hepatic I/R.

The levels of adiponectin are also reduced in obese subjects[8,22-25] and in experimental fatty liver models, regardless of the type of steatosis (diet- or alcohol-induced).[26-28] Indeed, in a cohort of 68 obese individuals, the serum levels of adiponectin significantly predicted hepatic steatosis and hepatic damage.[29,30] Research aimed at identifying prognostic factors in liver transplantation are both necessary and relevant. Further investigations are required to determine whether the measurement of serum adiponectin, a noninvasive tool, could predict the severity of steatosis and liver damage and contribute to the identification of high-risk steatotic liver donors. The decision to implant or reject a steatotic liver is difficult because of the risk of impaired graft function and even failure after implantation. What amounts and types of fat represent significant risks for primary nonfunction of the graft remain matters of debate. The assessment of donor liver fat is a difficult task for the transplant team because of large inconsistencies in the qualitative and quantitative measurement of fat deposits in the liver.[31-34]

ROLE OF ADIPONECTIN IN HEPATIC I/R INJURY

Adiponectin is considered a potential regulator of hepatic steatosis[18,30,35,36] and a negative modulator of the systemic and hepatic inflammation[18,28,30,36-40] that characterizes different liver diseases. Because of the anti-obesity and anti-inflammatory properties of adiponectin, the use of adiponectin could be a promising strategy for treating steatotic livers subjected to I/R. The role of fatty infiltration and inflammation in the vulnerability of steatotic livers to I/R is well established.[1-5] However, this role may not be applicable to hepatic I/R (as discussed later). To the best of our knowledge, only 2 studies have reported a role for adiponectin in steatotic livers subjected to I/R, and these studies were based on the pharmacological modulation of adiponectin.[10,16] Massip-Salcedo et al.[10] showed injurious effects of adiponectin on steatotic livers subjected to warm ischemia. Under these conditions, steatotic livers showed higher levels of adiponectin mRNA expression and higher adiponectin protein levels in comparison with nonsteatotic livers. A treatment with adiponectin silent small interfering RNA (siRNA) decreased oxidative stress and hepatic injury in steatotic livers. Peroxisome proliferator-activated receptor alpha (PPAR-α) agonists and ischemic preconditioning (PC) via PPAR-α inhibited mitogen-activated protein kinase expression after I/R. This in turn inhibited the accumulation of adiponectin in steatotic livers and reduced its negative effects on oxidative stress and hepatic injury. In a recent study,[17] the addition of melatonin to the Institut Georges Lopez I preservation solution reduced hepatic injury, and its benefits correlated with a reduction in adiponectin levels. The relevance of the changes in adiponectin induced by melatonin were not investigated. However, another study of small fatty grafts by Man et al.[16] demonstrated high serum adiponectin levels after transplantation. A treatment with adiponectin exerted (1) anti-inflammatory effects that down-regulated tumor necrosis factor α (TNF-α) mRNA and (2) vasoregulatory effects that improved the microcirculation. The anti-inflammatory effects of adiponectin also included the activation of cell survival signaling via the phosphorylation of Akt and the stimulation of nitric oxide (NO) production. Additionally, Man et al. demonstrated the anti-obesity and proliferative properties of adiponectin in small fatty transplants. The

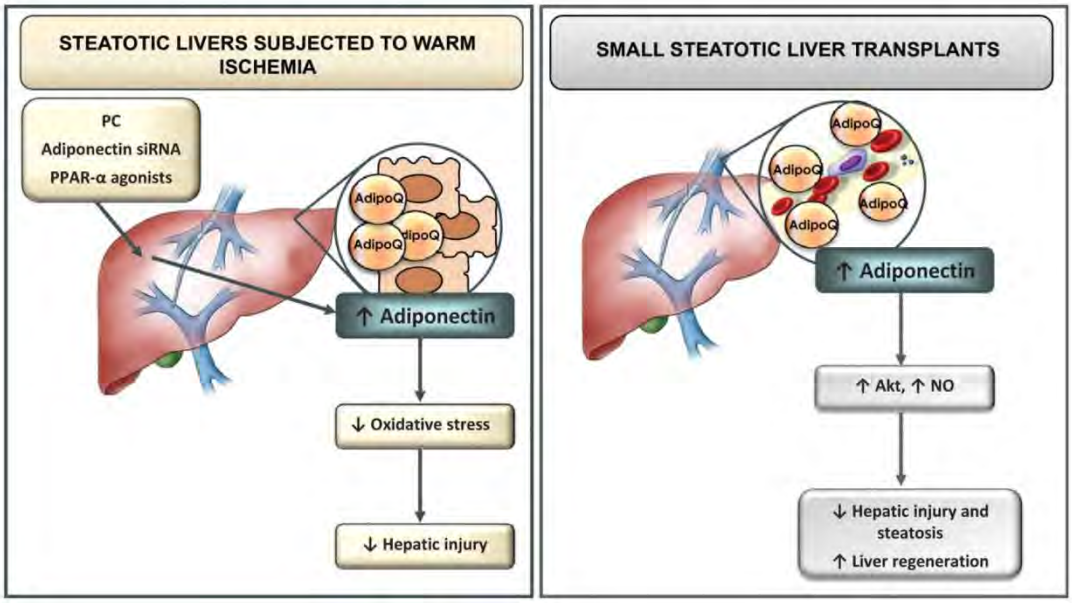

Figure 1. Roles of adiponectin in steatotic livers subjected to warm ischemia and in small steatotic liver transplants.

main differences in the roles of adiponectin in hepatic I/R are shown in Fig. 1.

Next, we discuss how adiponectin could affect the mechanisms involved in the vulnerability of steatotic livers to I/R (Fig. 2). Fatty degeneration induces a series of ultrastructural and biochemical alterations in both human and animal mitochondria. The lower adenosine triphosphate (ATP) and adenine nucleotide levels in steatotic livers preserved in University of Wisconsin solution could be caused by mitochondrial damage.[1,41,42] Caraceni et al.[43] reported that alterations in oxidative phosphorylation during preservation are greatly enhanced by fatty infiltration due to damage to respiratory chain complex I and F0F1-ATP synthase. Other studies have shown that during warm ischemia or transplantation, the level of mitochondrial uncoupling protein 2 (UCP2) is 4 to 5 times higher in steatotic livers versus nonsteatotic livers.[44-46] This finding has been associated with a reduced ability to synthesize ATP upon reperfusion.[44] Previous studies have indicated that steatotic livers have a reduced ability to respond to endoplasmic reticulum (ER) stress.[47] ER stress in steatotic livers activates the mitochondrial cell death pathway, which results in inflammation, apoptosis, and necrosis. Man et al.[16] found that UCP2 and fatty acid synthase (FAS) were activated in small fatty liver grafts after transplantation, and they promoted ATP depletion and necrosis. An adiponectin treatment reduced UCP2 and FAS levels (Fig. 2) and increased ATP levels after liver transplantation.

Cell death can occur via necrosis or apoptosis, and the intracellular ATP level appears to play a role as a putative apoptosis/necrosis switch: when ATP depletion is severe, necrosis ensues before the activation of the energy-requiring apoptotic pathway.[4,48] In steatotic liver grafts subjected to 6 hours of cold ischemia, necrosis was the predominant process of cell death, and no signs of apoptosis were found.[49,50] Because apoptosis is an energy-requiring process, the impaired maintenance of ATP levels observed after reperfusion in steatotic livers subjected to long periods of cold ischemia may be linked to a failure to induce apoptosis. Not surprisingly, previously reported data indicate that necrosis rather than apoptosis is the predominant process by which fatty livers undergo cell death.[49-51] However, Man et al.[16] reported that small steatotic liver grafts subjected to 40 minutes of cold ischemia underwent apoptosis, which was reduced after a pretreatment with adiponectin. Because of the short cold ischemia times, the ATP depletion may not have been sufficiently severe to induce necrosis, and this may have allowed apoptosis to take place.

Massip-Salcedo et al.[10] showed that adiponectin regulation reduced oxidative stress in steatotic livers subjected to warm ischemia, although the way in which adiponectin affected oxidative stress was not evaluated. In the same experimental model of warm ischemia, higher interleukin-1β (IL-1β) levels and lower IL-10 levels were detected after reperfusion in steatotic livers versus nonsteatotic livers.[52] This imbalance between proinflammatory and anti-inflammatory ILs increased the oxidative stress and decreased the tolerance of steatotic livers for I/R (Fig. 2). Given these data and the fact that adiponectin regulates IL-10 and IL-1R antagonists in different

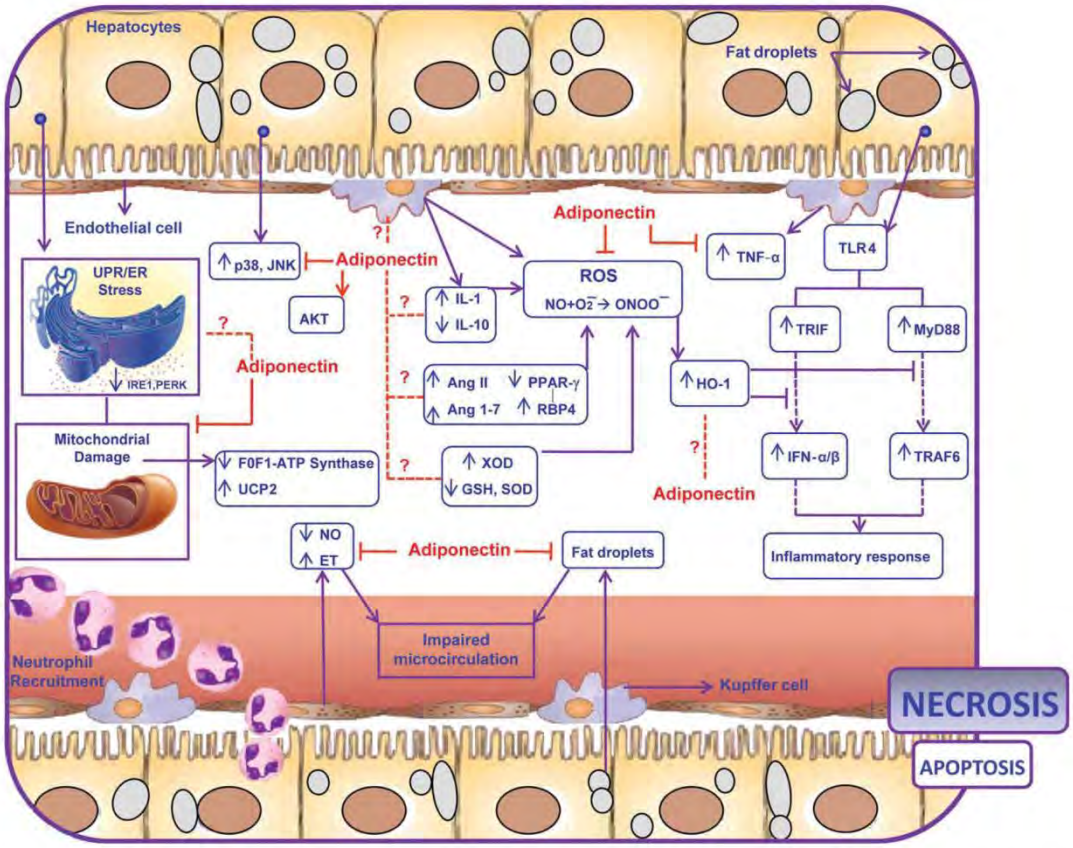

Figure 2. I/R injury mechanisms in steatotic livers that could be modulated by adiponectin. Abbreviation: IRE1, Inositol-requiring enzyme 1; PERK, protein kinase-like endoplasmic reticulum kinase.

cells,[6,53] we think that it is possible that adiponectin has effects on ILs in steatotic livers subjected to I/R. The effects of adiponectin on oxidative stress in small steatotic liver grafts were not evaluated by Man et al.[16] However, the possibility that adiponectin could regulate Kupffer cell activation should be considered. Indeed, in an experimental model of small liver transplants similar to the model described by Man et al., the main source of reactive oxygen species (ROS) was Kupffer cells, whereas xanthine dehydrogenase (XDH) and xanthine oxidase (XOD) played a minor role.[54] XDH and XOD are crucial for cold ischemia periods (6-8 hours)[55] that are longer than those described for small steatotic grafts (40 minutes). In addition to the high ROS level–generating system found in steatotic liver grafts, this type of liver shows low levels of antioxidants such as glutathione (GSH) and superoxide dismutase (SOD).[50,56] The overexpression of SOD by gene therapy, tocopherol, and a GSH pretreatment reduced the damage to steatotic livers and increased their survival.[56-59] However, difficulties have been found during attempts to prevent I/R injury in steatotic livers with therapies aimed at inhibiting ROS production. Indeed, *N*-acetylcysteine, a GSH precursor, did not reduce ROS.[60] Additionally, steatotic livers might produce SOD/catalase-insensitive ROS, which may be involved in the mechanism of failure of steatotic livers after transplantation.[61] Moreover, gene therapy based on antioxidant overexpression is limited by the toxicity of the vectors.[2,62] Because of the effects of adiponectin on antioxidant systems in the liver,[6,63] adiponectin regulation could be an alternative way of increasing antioxidant defenses in steatotic livers, which may help to reduce the greater oxidative stress (Fig. 2). Previous studies under conditions of warm and cold ischemia have indicated that alterations in the renin-angiotensin (Ang) system, retinol-binding protein 4 (RBP4), and PPAR-γ contribute to oxidative stress and damage in steatotic livers.[49,64,65] Further research is required to determine whether adiponectin that accumulates in steatotic livers as a result of I/R (as seen under conditions of warm ischemia) is the cause or effect of such alterations.

In agreement with the studies of small steatotic liver grafts by Man et al.,[16] reductions in the hepatic microcirculation have been observed in fatty livers

from human donors and in experimental models of hepatic steatosis.[66-68] Man et al. reported that adiponectin reduced the expression of endothelin 1 (ET-1) and increased the expression of constitutive NO synthase after reperfusion in small steatotic liver grafts and thus potentially could ameliorate the microcirculatory disorders associated with hepatic I/R (Fig. 2). An imbalance between vasoconstrictors (eg, ET-1) and vasodilators (eg, NO) negatively affects the hepatic microcirculation.[1,69] In addition, the reduction in steatosis found in small steatotic liver grafts after a pretreatment with adiponectin could improve the hepatic microcirculation. It is well known that the accumulation of fat in the cytoplasm of hepatocytes is associated with an increase in the cell volume that reduces the size of the hepatic sinusoid space by 50% in comparison with a normal liver, and this may result in a partial or complete obstruction of the hepatic sinusoid space.[3,66-68] Massip-Salcedo et al.[10] did not investigate the effects of adiponectin on the hepatic microcirculation in steatotic livers subjected to warm ischemia. However, on the basis of previous studies, the potential modulation of the hepatic microcirculation induced by adiponectin could be less influential in steatotic livers subjected to warm ischemia. In fact, studies using the surgical procedure described by Massip-Salcedo et al. under conditions of warm ischemia have indicated that the failure of the hepatic microcirculation does not account for the greater damage in steatotic livers.

Different studies have demonstrated the proinflammatory role of TNF-α in the vulnerability of steatotic livers subjected to I/R.[50,70] However, in addition to its harmful effects, TNF-α plays a pivotal role in liver regeneration following transplantation.[71-73] Man et al.[16] indicated that adiponectin reduced TNF levels in steatotic liver grafts (Fig. 2). Nevertheless, the relevance of adiponectin-induced TNF reductions to damage, hepatic steatosis, and regeneration was not evaluated, and further studies will be required to answer this question.

Toll-like receptor 4 (TLR4) has been implicated as a mediator of steatotic liver damage after I/R.[74] The loss of TLR4 in steatotic livers from TLR4-knockout animals fed a high-fat diet reduces the levels of proinflammatory cytokines as well as liver injury and improves survival.[74] Although TLR4 signaling is relevant in hepatic I/R injury, there is some controversy about which pathway [the myeloid differentiation factor 88 (MyD88)–dependent signaling pathway or the toll/interleukin-1 receptor domain-containing adaptor inducing interferon-β (TRIF)/interferon regulatory factor 3 signaling pathway] is activated with hepatic I/R[75,76] (Fig. 2). Strategies aimed at blocking TLR4 signal amplification may be useful in steatotic liver transplantation because no TLR4 antagonists are commercially available or work well in vivo.[74] Such a strategy for blocking TLR4 signaling could involve adiponectin. Indeed, in a study using ethanol-fed rats, Mandal et al.[38] identified an adiponectin/heme oxygenase 1 (HO-1) pathway that mediates the anti-inflammatory effects of adiponectin by attenuating TLR4 signaling via MyD88 and TRIF pathways (Fig. 2). Further investigations are required to elucidate the involvement of HO-1 in the effects of adiponectin on TLR4. This possibility should be considered because in the same experimental model of I/R described by Massip-Salcedo et al.,[10] HO-1 was up-regulated in response to oxidative stress, and HO-1 activators reduced damage in steatotic livers subjected to warm ischemia.[77] The regulation of HO-1 by adiponectin could be interesting for liver surgery because most drugs used for the up-regulation of HO-1 under experimental conditions are not available for clinical use on account of their potential toxicity and undesirable or unknown side effects.[78]

Taken together, the aforementioned data indicate that the action mechanisms of adiponectin depend on the surgical conditions. As discussed previously, it is important to distinguish between the types of ischemia (warm and cold) because there is already some controversy about the pathophysiological mechanisms of cold ischemia, which may depend, for example, on the time. Moreover, the adiponectin data reported for these experimental models of hepatic I/R[10,16,17] should not be extrapolated to cadaveric organ transplantation. In the case of small liver transplants, the liver regeneration inherent in the surgical procedure and the mechanism of hepatic damage due to the removal of hepatic mass should be considered.[1,64,79] For small liver grafts (which are relatively common) and under conditions of warm ischemia, the periods of ischemia range from 40 to 60 minutes; this range may not be accurate for cadaveric donor liver transplantation. Additionally, in experimental models of the perfused liver, the liver is isolated from the influence of blood and other tissues, and this is not the case with cadaveric donor transplantation.

The pharmacological strategies aimed at modulating adiponectin are irrelevant for nonsteatotic livers subjected to warm ischemia. Findings such as these must be considered when the same pharmacological strategies are being applied indiscriminately to steatotic and nonsteatotic livers because the effects may be very different. Drugs based on adiponectin regulation have the potential to increase the number of organs suitable for transplantation because these drugs may improve the outcomes for marginal grafts that otherwise would not be transplanted. In addition, drugs based on adiponectin regulation could reduce the sensitivity of other marginal grafts, such as liver grafts from aged donors, to I/R injury. Indeed, aged donors have an increased incidence of steatosis, which may favor cold preservation injury.[1,5,80] Alterations in the activation of inflammatory transcription factors and the expression of cytoprotective proteins, increased levels of intracellular oxidants, and decreased mitochondrial function also characterize many age-related diseases.[1,81] However, because of the different results reported to date for hepatic I/R,[10,16,17] it is difficult to determine whether we should aim to inhibit adiponectin or administer adiponectin

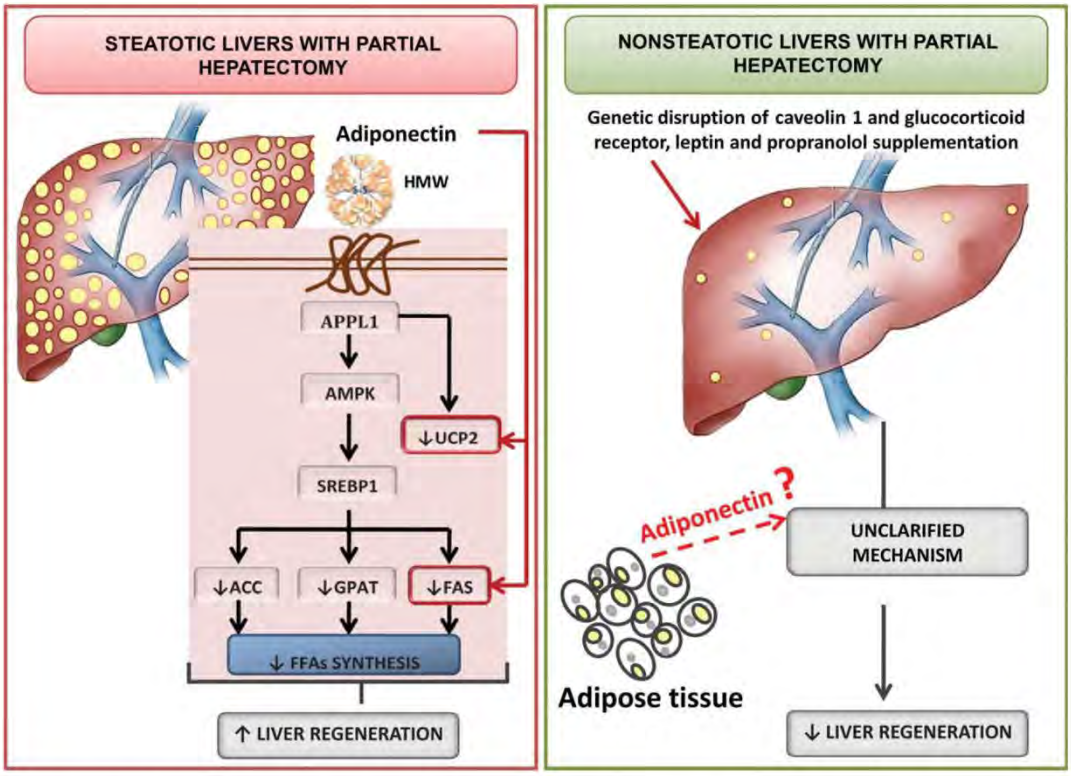

Figure 3. Effects of different treatments that reduce steatosis during liver regeneration in steatotic and nonsteatotic livers with partial hepatectomy.

to protect steatotic livers against cold ischemia associated with transplantation.

EFFECTS OF ADIPONECTIN ON STEATOSIS AND REGENERATION WITH HEPATIC I/R

According to previously reported data indicating that steatosis is a risk factor in liver surgery,[1-5] strategies aimed at reducing steatosis could increase the tolerance of steatotic livers for I/R. There is considerable evidence that liver regeneration is impaired in certain genetic models in which the liver contains excess fat.[16,64,82] For example, steatotic livers from obese mice exhibited defective liver regeneration and high mortality rates after partial hepatectomy.[82] Similarly, impaired liver regeneration was observed in steatotic livers versus nonsteatotic livers undergoing partial hepatectomy with vascular occlusion.[64] Man et al.[16] found that in small liver grafts, a pretreatment with adiponectin reduced steatosis and thus improved liver regeneration. Adiponectin down-regulated the expression of FAS and UCP2 and increased hepatic ATP levels[16] (Fig. 3). On the other hand, drugs that reduce hepatic steatosis should be considered with caution in clinical liver surgery because other studies (mainly involving nonsteatotic livers) have indicated that genetic or pharmacological approaches that reduce the accumulation of lipids may also hinder liver regeneration.[82-85] It is known that during early regeneration, the liver accumulates fat.[82-84] Interventions associated with the disruption of this transient hepatic accumulation of fat during early liver regeneration, including the genetic disruption of caveolin 1 or hepatic glucocorticoid receptor expression and leptin or propranolol supplementation, are associated with impaired regeneration in experimental models of partial hepatectomy[82,84,85] (Fig. 3). The specific molecular basis for these observations remains unknown. The authors suggested that hepatic steatosis may represent a required component for liver regeneration following partial hepatectomy because lipid droplets supply the energy needed by remaining hepatocytes to rebuild the liver.[83,84] It also has been suggested that catabolism of existing peripheral adipose stores followed by the hepatic accumulation of systemically derived fat may promote liver regeneration.[83,86] Indeed, in mice with fatty liver dystrophy and thus diminished peripheral adipose stores, the early

TABLE 1. Effects of Strategies That Regulate Adiponectin in Experimental Models and in Patients

Treatment	Experimental Models/Patients	Effects
Adiponectin[16]	Small steatotic liver grafts	↑ 7-day survival rate, ATP, Akt, NO, microcirculation, and regeneration ↓ Triglycerides, UCP2, ET-1, TNF-α, FAS, and hepatic injury
Adiponectin siRNA, PC, or PPAR-γ agonists[10]	Steatotic livers subjected to warm ischemia	↓ Adiponectin, inflammation, oxidative stress, and hepatic injury
Rimonabant[35,88]	Zucker fa/fa rats (10-11 weeks old)	↑ Adiponectin and high-density lipoprotein/low-density lipoprotein ratio ↓ Steatosis, hepatic injury, and TNF-α
Sibutramine[35,89]	Obese patients	↑ Adiponectin and weight loss
Thiazolidinediones[35,90-92]	Patients with nonalcoholic steatohepatitis or models of nonalcoholic fatty liver disease	↑ Adiponectin ↓ Steatosis, inflammation, and hepatic injury
Fibrates[35,93]	BKSCg mice and BKSCg db/db mice	↑ Adiponectin, PPAR-α, and PPAR-γ ↓ Steatosis
Atorvastatin[35,94]	Patients with nonalcoholic fatty liver disease	↑ Adiponectin ↓ Cholesterol and hepatic injury
Resveratrol[26]	C57BL/6J mice fed an ethanol-containing low-fat diet	↑ Adiponectin ↓ Hepatic lipid accumulation
S-Adenosyl methionine[26,95]	Yucatan micropigs fed ethanol and a folate-deficient diet	↑ Adiponectin, SREBP1, FAS, and ACC ↓ Steatosis, adiponectin receptor 1, and TNF-α
Betaine[26,96]	C57BL/6 mice fed a high-fat diet and ethanol	↑ Adiponectin ↓ Steatosis, hepatic triglycerides, ER stress, and hepatic injury
Ursodeoxycholic acid plus vitamin E[35,97]	Patients with nonalcoholic steatohepatitis	↑ Adiponectin ↓ Glucose, steatosis, and hepatic injury
Olmesartan[35,98]	Diabetic C57BL/6J mice and C57BL/6J mice fed a high-fat diet	↑ Adiponectin ↓ Steatosis and fibrosis
Metformin[35,99]	Obese leptin-deficient mice	↑ Adiponectin and AMPK ↓ Steatosis and TNF-α
Spironolactone[35,100]	Diabetic patients	↑ Adiponectin and visfatin ↓ TNF-α
Anti-TNF[35]	Patients with nonalcoholic steatohepatitis	↑ Adiponectin
Oxyntomodulin[35,101]	Obese patients	↑ Adiponectin and weight loss
Orlistat[35,102]	Patients with nonalcoholic steatohepatitis	↑ Adiponectin, resistin, and TNF-α ↓ Steatosis, cholesterol, and hepatic injury
Valsartan[35,103]	Hypertensive patients with metabolic syndrome	↑ Adiponectin ↓ Triglycerides and total cholesterol
Candesartan or temocapril[35,104,105]	Hypertensive patients	↑ Adiponectin ↓ Blood pressure and FFA levels
Irbesartan or telmisartan[35,106]	Diabetic patients with metabolic syndrome	↑ Adiponectin ↓ Blood pressure, resistin, and triglycerides
Fosinopril[35,107]	Diabetic rats	↑ Adiponectin ↓ Steatosis

↑, increases; ↓, decreases.

hepatic fat levels were reduced after partial hepatectomy, and liver regeneration was impaired.[86] Future analyses should address whether the requirement for systemic adipose stores during normal liver regeneration is based on the role of adipose tissue as a source of metabolic fuel for regeneration, on its role as a lipid precursor for new membrane synthesis, or on its role as a specific signal initiating the regenerative response itself (or perhaps on all of these). Adipose tissue–derived hormones such as adiponectin have been identified as regulators of liver regeneration[86,87] (Fig. 3). One question is how much steatosis should be reduced in steatotic livers to protect this type of liver; as mentioned previously, a reduction in the hepatic fat content of nonsteatotic livers might be associated with impaired regeneration. Fatty droplets might supply the energy required for liver regeneration. Another question is whether we should reduce steatosis before the surgical procedure and, therefore, avoid the vulnerability of steatotic livers to I/R or instead use drugs aimed at reducing the levels of hepatic triglycerides during surgery and thus conserve the energy required for liver regeneration. Moreover, future studies should examine the effects of adiponectin on steatosis in cadaveric donor livers. In contrast to small liver grafts, hepatic regeneration is not a major issue; I/R injury is the main problem because of differences in the periods of cold ischemia.

THERAPEUTIC STRATEGIES BASED ON THE REGULATION OF ADIPONECTIN IN HEPATIC I/R

The effects of adiponectin on hepatic I/R and drugs that could have beneficial effects on steatosis and inflammation by increasing adiponectin levels are listed in Table 1.[10,16,26,35,88-107] Whether these approaches can be translated into treatments for clinical liver surgery remains unknown. For example, sibutramine should not be applied in clinical liver surgery because of its potential side effects on hypertension.[35,108] The development of hypertension after liver transplantation is nearly universal and reflects several pathogenic mechanisms.[109] Despite the potentially beneficial role of PPAR-α agonists, they should be used judiciously. Their short-term administration in humans (1-10 days) is unlikely to produce permanent genotoxic effects. However, long-term exposure to these drugs, which would be required to reduce hepatic steatosis, can result in oxidative DNA damage, among other effects.[110] Hepatic failure has been observed after the administration of these thiazolidinediones, and some case reports of acute hepatotoxicity attributed to rosiglitazone have been published; there has been 1 death.[111] The toxicity is thought to be mainly metabolic and idiosyncratic, although in some cases a possible immunological mechanism has been implicated.[111,112] Resveratrol at a high dose has been found to be a pro-oxidant and to aggravate liver injury, and experiments for devising a pharmaceutical form appropriate for clinical use are in progress.[113]

Further investigations are required to optimize these treatments because long-term therapy appears to be necessary for the desired effects. For example, the pretreatment times range from 2 to 4 weeks for betaine, metformin, oxyntomodulin, temocapril, candesartan, and quinapril,[96,99,101,104,105] from 6 to 12 weeks for sibutramine, rosiglitazone, fibrates, spironolactone, valsartan, and ramipril,[89,93,100,103] and from 14 to 17 weeks for *S*-adenosyl methionine and olmesartan.[95,98] There are obvious difficulties with the feasibility of long-term drug administration for some I/R processes; this is particularly true for liver transplantation with a cadaveric donor because there is very little time to pretreat the donor with drugs for this emergency procedure. Adiponectin could be a target in liver surgery. Indeed, the pretreatment times were markedly shorter for adiponectin (24 or 48 hours) versus other drugs (Table 1).

CONCLUSIONS AND PERSPECTIVES

Because of the anti-inflammatory and anti-obesity properties of adiponectin, it could be a target in steatotic liver transplantation. However, because of the different reported results with hepatic I/R injury, future investigations are required to determine whether it is necessary to inhibit or activate adiponectin in the setting of liver transplantation to protect livers against I/R injury. Before a complete definition of a successful therapeutic strategy based on adiponectin regulation is formed, several additional points need to be addressed. The biologically active forms of adiponectin must be identified in detail, and comparative studies of the different isoforms of the adipokine in hepatic I/R are required. The effects of adiponectin on the pathway involved in the inflammatory process and lipid metabolism have only just been mapped. The response of adiponectin depends on the surgical procedure. Moreover, the responses of different types of livers to adiponectin stimulation might differ and involve different signal transduction pathways that are at present marginally understood. To avoid possible side effects, investigations of the mechanisms of adiponectin will be required to develop novel targets and new drugs for steatotic liver transplantation before its therapeutic use. The large-scale production of the native forms of full-length adiponectin (properly assembled into supramolecular complexes) or antibodies that block its action might be a challenging task.

The potential applications of drugs that regulate adiponectin are numerous in liver surgery, and they may provide a novel therapeutic approach to clinical conditions in which I/R injury and liver regeneration occur (including hepatic resection). In addition, these drugs have the potential to increase the number of organs suitable for transplantation because they may improve the outcomes for marginal grafts that

otherwise would not be transplanted and lead to new possibilities for small steatotic liver transplants.

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Review Article

The Current Knowledge of the Role of PPAR in Hepatic Ischemia-Reperfusion Injury

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Strategies to improve the viability of steatotic livers could reduce the risk of dysfunction after surgery and increase the number of organs suitable for transplantation. Peroxisome proliferator-activated receptors (PPARs) are major regulators of lipid metabolism and inflammation. In this paper, we review the PPAR signaling pathways and present some of their lesser-known functions in liver regeneration. Potential therapies based on PPAR regulation will be discussed. The data suggest that further investigations are required to elucidate whether PPAR could be a potential therapeutic target in liver surgery and to determine the most effective therapies that selectively regulate PPAR with minor side effects.

1. Introduction

Liver transplantation has evolved as the therapy of choice for patients with end-stage liver disease. However, the waiting list for liver transplantation is growing at a rapid pace, whereas the number of available organs is not increasing proportionately. The potential use of steatotic livers, one of the most common types of organs in marginal donors, for transplantation has become a major focus of investigation. However, steatotic livers are more susceptible to ischemia-reperfusion (I/R) injury, and the transplantation of steatotic levels results in a poorer outcome than that of nonsteatotic livers. Indeed, the use of steatotic livers for transplantation is associated with an increased risk of primary nonfunction or dysfunction after surgery [1, 2]. In hepatic resections, the operative mortality associated with steatosis exceeds 14%, compared with 2% for healthy livers, and the risks of dysfunction after surgery are similarly higher [2, 3]. Despite advances aimed at reducing the incidence of hepatic I/R injury (summarized in earlier reviews) [1, 2], the results to date are inconclusive. In this paper, we review the peroxisome proliferator-activated receptor alpha (PPARα) and PPARγ signaling pathways in steatosis, inflammation and regeneration, three key factors in steatotic liver surgery [1–5]. Our review of the different strategies pursued to regulate PPAR in liver diseases may motivate researchers to develop effective treatments for steatotic livers in patients undergoing I/R. The potential clinical application of strategies that regulate PPAR in the setting of steatotic liver surgery is also discussed.

2. Characteristics of PPAR

PPARs belong to the hormone nuclear receptor superfamily and consist of three isoforms: PPARα, PPARγ, and PPARβ/δ. Of these, our group and others have demonstrated that PPARα and PPARγ are important regulators of postischemic liver injury [1, 2, 6, 7] that exert their effects on steatosis and inflammation, which is inherent in steatotic liver surgery [8–12].

Previous results indicate that the presence of fatty infiltration by itself in the liver (without any surgical intervention) does not induce changes in PPARα or PPARγ levels, as no differences were observed in the levels of these transcription factors between steatotic and nonsteatotic livers of

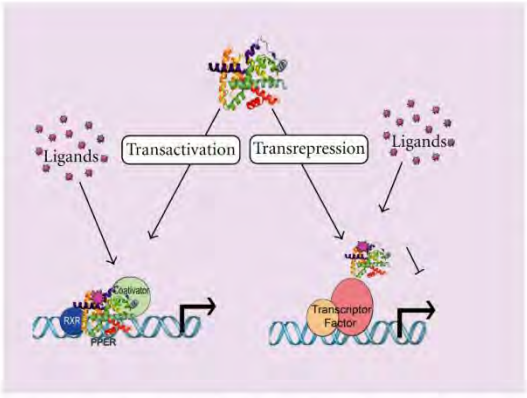

FIGURE 1: Basic mechanism of PPAR action. Receptor X retinoide, RXR; PPAR-response element, PPER.

a sham group of Zucker rats [13, 14]. These results contrast reports from the literature indicating high or low PPARγ levels in steatotic livers compared with those in nonsteatotic livers [15, 16]. These different results can be explained, at least in part, by differences in the level of PPARγ regulation between rats and mice [17], the different obesity experimental models evaluated, and the degree of steatosis. We reported that PPARγ expression levels in nonsteatotic livers during liver transplantation were similar to those observed in the sham group. However, increased PPARγ levels were observed in steatotic liver grafts [14, 18]. Thus, steatotic liver grafts are more predisposed to overexpress PPARγ. This is in line with clinical studies, in which PPARγ was upregulated in the livers of obese patients with nonalcoholic fatty liver disease (NALFD) [19]. Additionally, differences in PPARα expression were observed among different liver types. Indeed, steatotic livers are more predisposed to downregulate PPARα, when they are subjected to warm hepatic ischemia [13]. In line with these findings, PPARα is downregulated in the livers of obese patients with NALFD [20]. Findings such as these must be considered when applying the same pharmacological strategies indiscriminately to patients with steatotic and nonsteatotic livers because the effects may be very different.

PPARs can both activate and inhibit gene expression by two mechanisms: transactivation and transrepression. Transactivation is DNA- and ligand-dependent. PPARs activate transcription in a ligand-dependent manner by binding directly to specific PPAR response elements (PPREs) in target genes as heterodimers with retinoid X receptor (RXR). Agonist binding leads to the recruitment of coactivator complexes that modify the structure of chromatin and facilitate the assembly of the general transcriptional machinery at the promoter [21]. Transrepression is ligand-dependent and may explain the anti-inflammatory actions of PPARs [22]. PPARs repress transcription by antagonizing the actions of other transcription factors [21] (see Figure 1). Physiologically, PPAR-RXR heterodimers may bind to PPREs in the absence of a ligand. Although the transcriptional activation depends on the ligand-bound PPAR-RXR, the presence of unliganded PPAR-RXR at a PPRE has effects that vary depending on the promoter context and cell type [22]. Further investigations on the structures of PPARs and the mechanisms by which PPARs regulate gene transcription may be useful for designing certain strategies, such as the use of PPAR antagonists or agonists. As shown in the following sections, the currently used pharmacological strategies aimed at regulating PPAR could not be incorporated into liver surgery due to their potential side effects.

Given the antiobesity and anti-inflammatory properties of PPARα and PPARγ [8–12], pharmacological interventions targeting these transcription factors could be a promising strategy to treat hepatic steatosis in patients undergoing I/R. However, as shown in Figure 1, the effects of pharmacological strategies aimed at modulating PPARs depend on the type of ischemia (cold or warm ischemia), the length of ischemia, and the type of the liver (nonsteatotic or steatotic liver).

3. Effect of PPAR on Hepatic I/R

To the best of our knowledge, few studies have examined both the I/R-inducedexpression of hepatic PPARα and the potential benefits of PPARα agonists under these conditions. According to previous studies by our group, PPARα mRNA and protein levels in nonsteatotic livers during I/R were similar to those of the sham group, and PPARα did not play a crucial role in I/R injury in nonsteatotic livers [13]. This contrasts studies published by Okaya and Lentsch [23] and Xu et al. [24], who reported the benefits of PPARα agonists in postischemic liver injury. The protective effects were possibly associated with reductions in neutrophil accumulation, oxidative stress, and tumor necrosis factor (TNF) and interleukin-1 (IL-1) expression (Figure 2). Although the dose and pretreatment time of the PPARα agonist WY-14,643 were similar in both studies, Okaya and Lentsch [23] and Xu et al. [24], reported an ischemic period of 90 min [23, 24]; our ischemic period was 60 min, which is the ischemic period currently used in liver surgery [13]. Thus, 60 min of ischemia appears insufficient for inducing changes in PPARα levels in nonsteatotic livers. In nonalcoholic steatohepatitis (NASH) and simple steatosis, treatment of mice with the PPAR activator Wy-14,643 protects steatotic livers against I/R injury, and the benefits of this treatment potentially occur through the dampening of adhesion molecule and cytokine responses and activation of nuclear factor kappa B (NF-κB) and IL-6 production [25]. In steatotic livers undergoing warm ischemia, PPARα agonists can limit the damage induced by I/R. PPARα agonists as well as ischemic preconditioning (PC) through PPARα inhibited mitogen-activated protein kinases (MAPK) expression following I/R (Figure 2). This in turn inhibited adiponectin accumulation in steatotic livers and adiponectin worsening effects on oxidative stress and hepatic injury [13]. Given these data, PPARα regulation could be an alternative method for reducing the greater oxidative stress incurred by steatotic livers. Indeed, preventing I/R injury in steatotic livers via therapies aimed at inhibiting reactive oxygen species (ROS) production has proven difficult. Steatotic livers might produce SOD/catalase-insensitive ROS, which

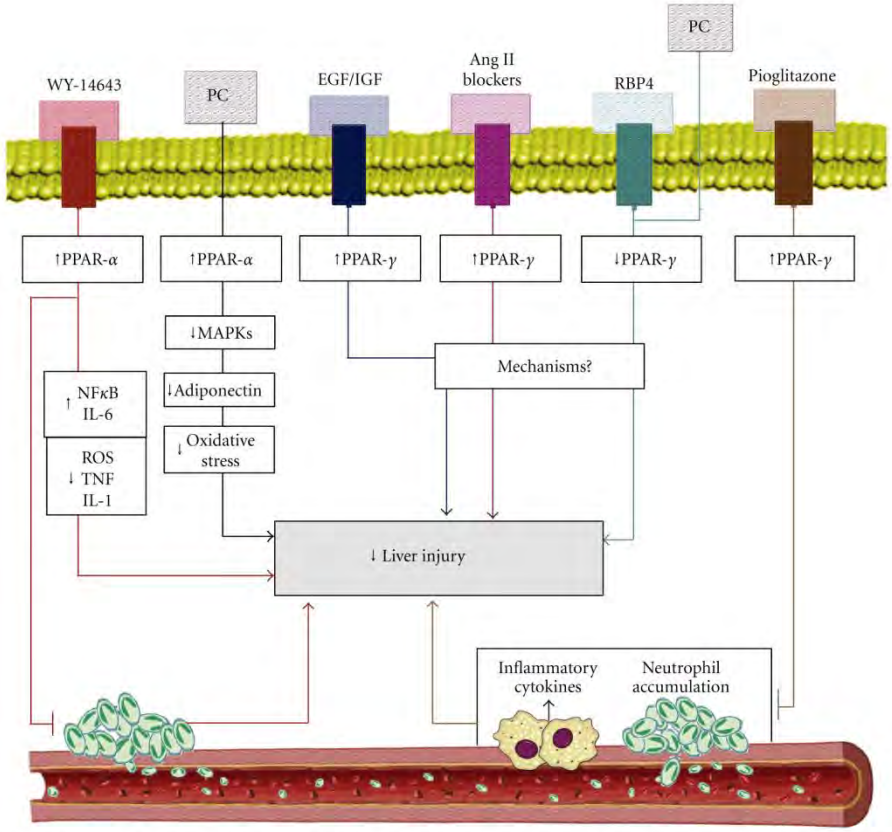

FIGURE 2: PPAR and hepatic I/R injury. Angiotensin II, Ang II; epidermal growth factor, EGF; insulin-like growth factor, IGF; interleukin-6, IL-6; mitogen-activated protein kinases, MAPKs; nuclear factor kappa B, NFκB; PPARα agonist; pioglitazone, peroxisome proliferator-activated receptors, PPAR; ischemic preconditioning, PC; retinol binding protein, RBP4, PPARα agonist; Wy-14,643.

may be involved in the mechanism of failure of steatotic livers after transplantation [26]. Moreover, gene therapy based on antioxidant overexpression is limited by the toxicity of the vectors [2, 27]. In a recent study of nonsteatotic livers undergoing warm hepatic ischemia, the dietary supplementation with n-3 polyunsaturated fatty acids (PUFAs) increased hepatic n-3 PUFA content and reduced hepatic n-6/n-3 PUFA content. This was associated with PPARα upregulation, which in turn reduced NF-κB signaling and oxidative stress, leading to a reduced inflammatory response [28].

The function of PPARγ in hepatic I/R injury is unclear. Previous results in liver transplantation studies indicated that I/R did not induce changes in PPARγ expression in nonsteatotic livers, and consequently, strategies based on PPARγ regulation had no effect on hepatic injury [14]. These results were different from those observed in nonsteatotic livers under warm ischemia conditions [6]. In that study, treatment with pioglitazone, a PPARγ agonist, significantly inhibited hepatic I/R injury (Figure 2). The protective effect was associated with the downregulation of several proinflammatory cytokines and chemokines and neutrophil accumulation [7]. This is in line with other results indicating that PPARγ-deficient mice displayed more severe injuries than untreated mice under warm ischemia conditions [6]. Furthermore, pioglitazone treatment inhibited apoptosis and significantly improved the survival of mice in a lethal model of hepatic I/R injury [7]. Previous studies indicated that PPARγ activation inhibits the release of TNFα, IL-1, and IL-6 by macrophages [29, 30], which could be of interest in steatotic livers. Indeed, under warm hepatic ischemia, higher IL-1 and lower IL-10 levels were detected in steatotic livers after reperfusion than in nonsteatotic livers [31]. This imbalance between pro- and anti-inflammatory ILs increased oxidative stress and decreased the tolerance of steatotic livers to I/R. In addition, different studies have reported proinflammatory and anti-inflammatory roles of TNF-α and IL-6, respectively, in the vulnerability of steatotic livers undergoing I/R [2, 32].

Previous results indicated that PPARγ activation in hepatocytes by rosiglitazone treatment increases autophagy and protects against hepatic I/R injury. Autophagy is

an evolutionarily conserved cellular process for recycling of old proteins and organelles via the lysosomal degradation [33]. Thus, these results suggest that PPARγ has anti-inflammatory properties and therefore may be relevant during hepatic I/R injury. In line with these data, PPARγ upregulation is a key mechanism of the benefits of different pharmacological or surgical strategies for steatotic livers undergoing I/R. Thus, some results based on isolated perfused livers indicated that the addition of growth factors (epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-I)) to University of Wisconsin (UW) preservation solution protected steatotic livers due to PPARγ overexpression [34]. Similarly, EGF pretreatment mediated by PPARγ overexpression protected steatotic livers undergoing warm ischemia [35] (Figure 2). Moreover, in warm hepatic ischemia, PPARγ upregulation was a key mechanism of the benefits of pharmacological blockers of angiotensin II (angiotensin-converting-enzyme (ACE) inhibitors and Ang II receptor antagonists) on steatotic livers [36]. However, the role of PPARγ in hepatic I/R injury could depend on the surgical conditions, as a recent study of liver transplantation indicated that treatment with a PPARγ antagonist was effective in steatotic livers, suggesting a detrimental role of PPARγ under these conditions [14]. In line with this finding, PPARγ inhibition was a key mechanism of the benefits of RBP4 treatment and PC on steatotic liver grafts [14]. Considering these results, drugs targeting PPARγ regulation can potentially increase the number of organs suitable for transplantation, as these drugs can improve the outcome for marginal grafts that would not otherwise have been transplanted. However, the data on PPARγ reported in steatotic liver transplantation models with standard liver graft sizes should not be extrapolated to small-size steatotic liver grafts. In the case of small liver transplants, the liver regeneration inherent in this surgical procedure and the mechanism of hepatic damage derived from the removal of hepatic mass should be considered [1, 31, 36]. In small liver grafts the periods of ischemia ranged 40–60 min, whereas the periods of ischemia ranged 6–8 hours for cadaveric donor liver transplantation.

4. Effect of PPAR on Hepatic Steatosis

Numerous studies suggest that the actions of PPARα can prevent steatosis. Mice deficient in PPARα develop hepatic steatosis when fasted or fed a high-fat diet [37, 46, 57]. Treatment with a PPARα agonist decreased hepatic steatosis in mice on a methionine- and choline-deficient (MCD) diet [37]. Activation of PPARα by the agonist Wy-14,643 ameliorated alcoholic fatty liver- and MCD-induced steatohepatitis [37, 38]. The critical role of PPARα in ameliorating steatosis is mediated through the regulation of a wide variety of genes involved in peroxisomal, mitochondrial, and microsomal FA β-oxidation systems in the liver [58]. When steatotic livers are submitted to certain stresses much as partial hepatectomy, the activation of PPARα by bezafibrate reduces the availability of FAs from circulation, reducing thus the hepatic sphingolipid synthesis [40] (see Table 1).

It is well known that n-3 PUFAs and their derivative FAs activate PPARα [59–61], which then heterodimerizes with RXR and liver X receptor, leading to the transcription of a large number of genes involved in lipid metabolism. It has been reported that n-3 PUFAs are more potent than the n-6 PUFAs as in vivo activators of PPARα [59]. In addition, PUFA metabolites such as eicosanoids or oxidized FAs have one to two orders of magnitude greater affinity for PPARα and are consequently far more potent transcriptional activators of PPARα-dependent genes [59].

The interaction of PPARα with its DNA recognition site is markedly enhanced by ligands such as hypotriglyceridemic fibrate drugs, conjugated linoleic acid, and PUFAs [59]. The discovery of PPARα led quickly to the idea that PPARα was a "master switch" transcription factor that was targeted by PUFA to coordinately suppress genes encoding lipid synthesis proteins and to induce genes encoding lipid oxidation proteins [59]. In line with this idea, recent studies suggested that n-3 FAs serve as important mediators of gene expression, working via the PPARs to control the expression of the genes involved in lipid and glucose metabolism and adipogenesis [61]. Neschen et al. [62] demostrated that the administration of dietary fish oil (n-3) to rats increases the FA capacity of their livers through its ability to function as a ligand activator of PPARα and thereby induces the transcription of several gene-encoding proteins affiliated with FA oxidation. Of interest, other studies examining the effects of fish oil feeding on the expression of several genes of PPAR knockout mice clearly indicated that hepatic gene regulation by fish oil feeding involves at least two different pathways: PPARα-dependent and PPARα-independent pathways. Enzymes for peroxisomal (CYP4A2) and microsomal (AOX) oxidation are PPARα-dependent and upregulated by fish oil feeding, whereas those for lipid synthesis (FAS; S14) are PPARα-independent and downregulated. This indicates that the FA regulation of de novo hepatic lipogenesis and FA oxidation are not mediated through a common factor (e.g., PPARα) [61].

Given all these data into in account, the regulation of PPARα by PUFA, particularly n-3 PUFA and possibly conjugated linoleic acid, may offer an explanation for the reported benefits of these FAs in different pathologies.

In obese NAFLD patients, the increased production of ROS leads to the depletion of n-3 PUFAs due to enhanced lipid peroxidation. As PPARα is activated through direct binding to n-3 PUFA, liver PPARα function is compromised in obesity. This prevented the upregulation of genes involved in lipid transport, FA β-oxidation and thermogenesis, favoring FA and triacylglycerol synthesis over FA β-oxidation and thus promoting hepatic steatosis [20]. Thus, PPARα activation by n-3 PUFA supplementation ameliorated hepatic steatosis in obese NAFLD patients [20]. In line with this, NASH patients have low levels of circulating n-3 PUFA, with a consequent increase of the n-6/n-3 FA ratio and impaired PPARα activity in the liver [42, 43]. NASH patients treated with eicosapentaenoic acid (EPA) or n-3 PUFAs, a mixture of EPA and docosahexaenoic acid, exhibited improvements in hepatic steatosis and necroinflammation in humans and rats with NASH, probably due to the reduction of hepatic TNFα expression and improvement of insulin sensitivity [41–43]. Moreover, PUFAs activate PPARα, leading to

Table 1: Effect of strategies that regulate PPAR on hepatic injury, steatosis, and regeneration in experimental models and patients. Angiotensin II: Ang II; choline deficient: CD; epidermal growth factor: EGF; high-fat diet: HFD; insulin-like growth factor 1: IGF-1; methionine choline deficient: MCD; nonalcoholic Steatohepatitis: NASH; peroxisome proliferator-activated receptors: PPARs; polyunsaturated fatty acids: PUFAs; ischemic preconditioning: PC; retinol binding protein-4: RBP4.

PPARα					
PPARα activators					
Strategies	Time	Effect	Experimental model and patients	Steatosis and hepatic injury	Regeneration
WY-14,643 (30 μmol/kg/d) [17]	3 weeks	↑ PPARα	Obese Zucker rats	↑ β-oxidation of fatty acids	Not evaluated
WY-14,643 (180 μmol/kg/d) [17]	1 week	↑ PPARα	Ob/ob mice	↑ β-oxidation of fatty acids; ↓ triglycerides	Not evaluated
WY-14,643 (10 mg/kg) [23, 24]	1 h before ischemia	↑ PPARα	Mice or Rats; warm ischemia (90 min)	↓ hepatic injury	Not evaluated
WY-14,643 (10 mg/kg) [13]	1 h before ischemia	↑ PPARα	Zucker obese rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
WY-14,643 (10 mg/kg) [25]	10 days before surgery	↑ PPARα	Foz/foz mice; steatotic livers; warm ischemia (90 min)	↓ hepatic injury	↑ cell cycle entry
Wy-14,643 (0.1%) [37]	5 weeks	↑ PPARα	Mice fed MCD diet	↓ steatohepatitis	Not evaluated
Wy-14,643 (0.1%) [38]	12 days	↑ PPARα	Mice fed MCD diet	↓ steatohepatitis; ↑ hepatic fatty acid oxidation	Not evaluated
Bezafibrate [39]	5 weeks	↑ PPARα	Mice fed MCD	↓ hepatic triglycerides; ↑ hepatic fatty acid oxidation	Not evaluated
Benzafibrate (75 mg/kg) [40]	7 days	↑ PPARα	Rats; partial hepatectomy	↓ availability of fatty acids; sphingolipid synthesis	↓ liver regeneration
PC (5 min/10 min) [13]	Immediately before ischemia	↑ PPARα	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
n-3 PUFA (EPA (270 mg/kg) and DHA (180 mg/kg)) [28]	7 days	↑ PPARα	Sprague-Dawley rats; warm ischemia	↓ hepatic injury, inflammation, and oxidative stress	Not evaluated
EPA (2700 mg/d) [41]	1 year	↑ PPARα	NAFLD patients	↓ steatosis, hepatic injury, necroinflammation, and oxidative stress	Not evaluated
n-3 PUFA (1 g/day) [42]	1 year	↑ PPARα	NAFLD patients	↓ steatosis, hepatic injury, and necroinflammation	Not evaluated
n-3 PUFA (2 g/day) [43]	6 months	↑ PPARα	NAFLD patients	↓ steatosis, hepatic injury, necroinflammation, and hepatic injury	Not evaluated
n-3 PUFA (2 g, 3 times daily) [44]	24 weeks	↑ PPARα	NAFLD patients with hyperlipidemia	↓ steatosis and hepatic injury	Not evaluated
Ω-3 FA (5 mL, thrice daily) [45]	24 weeks	↑ PPARα	NAFLD patients with dyslipidemia	↓ steatosis and hepatic injury	Not evaluated
Atorvastatin (20 mg/daily) [45]	24 weeks	↑ PPARα	NAFLD patients with dyslipidemia	↓ steatosis and hepatic injury	Not evaluated
Orlistat (120 mg, thrice daily) [45]	24 weeks	↑ PPARα	NAFLD patients with dyslipidemia	↓ steatosis and hepatic injury	Not evaluated

Table 1: Continued.

PPARα knockout					
Strategies	Time	Effect	Experimental model	Steatosis and hepatic injury	Regeneration
PPARα-knockout [23]	—	↓ PPARα	PPARα-null mice Warm ischemia (90 min)	↑ hepatic injury	Not evaluated
PPARα-knockout [46]	—	↓ PPARα	PPARα-null mice fed HF diet	↑ hepatic β-oxidation	Not evaluated
PPARα-knockout [47]	—	↓ PPARα	PPARα-null mice Partial hepatectomy	Not evaluated	↓ liver regeneration
PPARγ					
PPARγ activator					
Strategies	Time	Effect	Experimental model	Steatosis and hepatic injury	Regeneration
Rosiglitazone (10 mg/kg) [6]	30 min before ischemia	↑ PPARγ	PPARγ^{+-} mice	↓ hepatic injury	Not evaluated
Rosiglitazone (2.5 μmol/kg/d) [17]	1 week	↑ PPARγ	Ob/ob mice	↓ triglycerides	Not evaluated
Rosiglitazone (3 mg/kg/day) [48]	5 weeks	↑ PPARγ	PPARγ$^{fl/fl}$ mice fed HFD diet	↑ steatosis	Not evaluated
Rosiglitazone (1 mg/kg/day) [49]	12 weeks	↑ PPARγ	Obese C57BL/6J mice	↑ steatosis	Not evaluated
Rosiglitazone (10 mg/kg) [50]	2 days before surgery	↑ PPARγ	Mice partial hepatectomy	Not evaluated	↓ hepatic regeneration
Troglitazone (0.1%) + adPPARγ [51]	adPPARγ (5th day) troglitazone (5 days)	↑ PPARγ	PPARα-null mice fed CD diet	↑ steatosis	Not evaluated
Pioglitazone (500 μg/Kg) [52]	8 weeks	↑ PPARγ	Rat fed liquid diet + alcohol	↓ liver injury	Not evaluated
Pioglitazone (30 mg) [53]	96 weeks	↑ PPARγ	Patients with NASH	↓ steatosis	Not evaluated
Pioglitazone (25 mg/kg/day) [54]	5 days before surgery	↑ PPARγ	KK-A^{Y}, mice partial hepatectomy	Not evaluated	↑ hepatic regeneration
Pioglitazone (20 mg/kg) [7]	1.5 h before ischemia	↑ PPARγ	Mice Warm ischemia (60 min)	↓ hepatic injury	Not evaluated
Ang II blockers Captopril (100 mg/kg) or PD123319 (30 mg/kg) [36]	Immediately before ischemia	↑ PPARγ	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
EGF and IGF-1 (10 μg/L) [34]	24 h in UW solution	↑ PPARγ	Obese Zucker rats; isolated liver perfused (24 h cold ischemia)	↓ hepatic injury	Not evaluated
EGF (100 μg/Kg) [35]	3 doses (every 8 h) starting before surgery	↑ PPARγ	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
IGF-I (400 μg/Kg) [35]	2 doses (every 12 h) starting before surgery	↑ PPARγ	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
Adenovirus PPARγ + rosiglitazone (50 mg/kg/day) [55]	8 weeks	↑ PPARγ	C57BL/6J mice fed MCD diet	↓ steatohepatitis and fibrosis	Not evaluated
PC (5 min/10 min) [36]	Immediately before ischemia	↑ PPARγ	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated

Table 1: Continued.

PPARγ inhibitor					
Strategy	Time	Effect	Experimental model	Steatosis and hepatic injury	Regeneration
GW9662 (1 mg/kg) [14]	1 h before surgery	↓ PPARγ	Liver transplantation (6 h cold ischemia)	Does not change in hepatic injury	Not evaluated
GW9662 (1 mg/kg) [14]	1 h before surgery	↓ PPARγ	Steatotic liver transplantation (6 h cold ischemia)	↓ hepatic injury	Not evaluated
GW9662 (1 mg/kg, 3 times/week) [55]	8 weeks	↓ PPARγ	C57BL/6J mice fed MCD diet	↑ steatohepatitis, fibrosis and hepatic injury	Not evaluated
RBP4 (150 μg/kg) [14]	30 min before surgery	↓ PPARγ	Steatotic liver transplantation (6 h cold ischemia)	↓ hepatic injury	Not evaluated
PC (5 min/10 min) [14]	Immediately before ischemia	↓ PPARγ	Steatotic liver transplantation (6 h of cold ischemia)	↓ hepatic injury	Not evaluated
PPARγ inhibitor					
Strategies	Time	Effect	Experimental model	Steatosis and hepatic injury	Regeneration
PPARγ-knockout [56]	—	↓ PPARγ	Liver-specific PPARγ-null mice	↓ steatosis	Not evaluated

increased FA β-oxidation; hence, they can shift the energy balance from storage to consumption [41, 43]. n-3 PUFAs have also been proved as safe and efficacious for patients with NAFLD associated with hyperlipidemia, as indicated by reduced hepatic damage and serum lipid levels [44]. In another study, the efficacy and safety of three hypolipidemic, agents in patients with NAFLD with dyslipidemia were evaluated. In this context, predominantly hypertriglyceridemic, hypercholesterolemic, and overweight patients were treated with n-3 FAs, atorvastatin, and orlistat, respectively. The three different groups of patients exhibited reduced hepatic damage, normalized of hepatic steatosis, and reduced serum lipids [45].

Considering that steatosis is a risk factor in liver surgery, strategies aimed to reduce steatosis could increase the tolerance of steatotic livers to I/R. There is considerable evidence that liver regeneration is impaired in certain genetic models in which the liver contains excess fat. For example, steatotic livers from Ob mice exhibit defective liver regeneration and high mortality following partial hepatectomy [63]. Similarly, impaired liver regeneration was observed in steatotic livers undergoing partial hepatectomy under vascular occlusion compared with that in nonsteatotic livers [31]. On the contrary, drugs that reduce hepatic steatosis, such as PPARα regulators, should be considered with caution in clinical liver surgery, as other studies indicate that genetic or pharmacologic approaches that reduce lipid accumulation may also hinder liver regeneration [63–66]. Thus, a question is to what degree should we reduce steatosis in steatotic livers to protect this type of liver. Another question is whether we should reduce steatosis before the surgical procedure and therefore avoid the vulnerability of steatotic livers to I/R, or in contrast, should we use drugs aimed at reducing hepatic triglycerides during surgery and thus conserve the energy required for liver regeneration. Moreover, research evaluating whether the short-term administration of PPARα agonists might alleviate hepatic steatosis in steatotic livers before I/R would be of interest for clinical practice because there are obvious difficulties concerning the feasibility of long-term PPARα agonist administration in some I/R processes, in particular liver transplantation from cadaveric donors, because this is an emergency procedure in which there is very little time to pretreat the donor with PPARα agonists.

Several studies attribute a causal role to PPARγ in the development of steatosis by mechanisms involving the activation of lipogenic genes and de novo lipogenesis [48, 51]. In accordance, targeted deletion of PPARγ in hepatocytes protects mice against diet-induced hepatic steatosis [67], suggesting a prosteatotic role of PPARγ. Similarly, mice with liver-specific PPARγ silencing are protected against hepatic steatosis [56]. Additionally, treatment of ob/ob mice with rosiglitazone increased liver steatosis [49]. By contrast, different results have been reported regarding the effect of PPARγ on hepatic steatosis. Indeed, PPARγ-deficient mice develop more severe MCD-induced NAFLD, whereas adenovirus-mediated PPARγ overexpression attenuated the progression of NASH [55]. In line with this finding, rosiglitazone treatment prevented the development of NASH in a model of MCD-treated mice [55], and similar results were obtained using the PPARγ agonist pioglitazone [52, 53]. These different results can be partially explained by differences in the studies such as the species, type of PPAR agonist, method to induce hepatic steatosis, the type of genetic strategy used to induce PPARγ overexpression or deficiency in PPARγ expression as well as differences in the pretreatment times of the drugs used (see Table 1).

5. Effect of PPAR on Hepatic Regeneration

Recent studies demostrated that liver regeneration is impaired in a number of animal models of fatty liver disease [68–73]. PPARα-null mice subjected to partial hepatectomy (PH) have an impaired ability to regenerate hepatic mass. Emerging evidence suggests that PPARα is a critical modulator of the energy flux important for the repair of liver damage. For example, hepatocytes in the periportal regions, which divide and replicate after PH, require mitochondrial oxidation of FAs to generate energy [74]. PPARα controls the constitutive expression of genes involved in mitochondrial FA oxidation, including carnitine palmitoyltransferase-1 [46, 75]. In mice deficient in PPARα, the impaired hepatic regeneration is also associated with the altered expression of genes involved in cell cycle control and cytokine signaling. Studies with PPARα agonists indicate that PPARα upregulates genes involved in the cycle cell (Ccnd1 and cMyc) as well as IL1r1 and IL-6r [76] (Figure 3).

It is well known that PPARα affects the transcription of a number of genes involved in lipid turnover and peroxisomal and mitochondrial β-oxidation, resulting in the generation of ATP, which is required to "fuel" liver repair and regeneration [76]. By contrast, in conditions in which PPARα function and/or expression is altered such as hepatic steatosis, and small-size liver grafts, FA metabolism is deviated toward the accumulation of inadequately metabolized fat, favoring ROS generation. Consequently, ATP production is decreased, and the demise of hepatocytes via necrotic cell death is increased, halting liver repair [77] (Figure 3). Accordingly, mice with targeted PPARα disruption exhibit increased inflammation and necrosis and delayed liver regeneration following partial hepatectomy [47].

Previous results indicate that the impaired liver regeneration of steatotic rats was partially due to PPARα downregulation through the AdipoR2 axis. The inhibition of PPARα signaling, increased triglyceride (TG) accumulation in hepatocytes and inhibited the expression of hepatic enzymes that contribute to FA oxidation (Figure 3). This was associated with increased lipid peroxidation and decreased antioxidant levels [78].

In contrast with the aforementioned data indicating the beneficial effects of PPARα on hepatic regeneration, a recent report indicated that PPARα activation by bezafibrate had negative effects on liver regeneration, which can be attributed to the inhibition of de novo sphingolipid synthesis [40]. Presumably, bezafibrate affects de novo sphingolipid synthesis by decreasing FA availability (Figure 3). The activation of PPARα by bezafibrate virtually obliterated the postoperative increase in plasma nonesterified FAs induced by PH. This can be explained by the inhibition of hormone-sensitive lipase activity in adipose tissue by PPARα ligands and their anti-inflammatory properties, which decrease the release of cytokines such as TNF and IL-6. Both events inhibited lipolysis in isolated white adipocytes, resulting in reduced FA release from extrahepatic sources after PH [40].

PPARγ activity is likely to be regulated during normal liver regeneration, and the disruption of this regulation could impair the regenerative response. Pioglitazone improved hepatic regeneration failure in obese mice. This effect was associated with reduced TNFα and IL-6 levels. Additionally, pioglitazone prevented the increased mRNA expression of signal transducer and activators of transcription-3 phosphorylation and suppressor of cytokine signaling-3 mRNA in the livers of obese mice [54]. However, inconsistent results have been obtained regarding the effect of PPARγ of liver regeneration. Indeed, rosiglitazone inhibited hepatocyte proliferation in mice undergoing partial hepatectomy by reducing p38 and cyclin expression [50] (see Figure 3).

On the basis of the inconsistent results reported to date on the role of PPAR in hepatic regeneration, it is difficult to discern whether we should attempt to inhibit PPAR or administer PPAR activators to promote liver regeneration in surgery.

6. Modulators of PPAR in Clinical Practice

Based on the data reported in experimental models (as reviewed above), different strategies (which have been summarized in Table 1) could exert effects on steatosis, inflammation, or regeneration by regulating PPAR. Whether these pharmacological approaches can be translated into treatments for clinical liver surgery remains unknown. For example, thiazolidinediones (TZDs) should not be applied in clinical liver surgery due to their potential side effects. TZDs (pioglitazone, troglitazone, and rosiglitazone) are synthetic PPARγ agonists that are widely used as antidiabetic agents [79–81]. However, prolonged treatment of obese and diabetic mice with TZDs resulted in the development of severe steatosis, which can lead to steatohepatitis and/or fibrosis. Troglitazone administration was associated with the development of idiosyncratic acute liver failure and was therefore withdrawn from clinical use [82, 83]. Hepatotoxicity has subsequently been reported in patients taking pioglitazone and rosiglitazone [83, 84]. These data provide support for current clinical practices in which these drugs are avoided or used judiciously in patients with known or suspected liver disease. Further experiments should be initiated to devise a pharmaceutical form appropriate for clinical use.

PPARα agonists are clinically and functionally relevant as fibrate therapeutics against hyperlipidemia and agents for reducing the complications of peripheral vascular disease in diabetic patients [85]. Despite their potentially beneficial roles, PPARα agonists should be used judiciously. Short-term administration in humans (1–10 days) would be unlikely to produce permanent genotoxic effects. However, long-term exposure to these drugs, which would be required to reduce hepatic steatosis, can result in oxidative DNA damage, among other effects [86–90] (Figure 4).

Further studies will also be required to elucidate whether growth factors, Ang II blockers, or RBP4 may be safer protective pharmacologic strategies for regulating PPAR in hepatic I/R injury in clinical practice (Figure 4). Nevertheless, none of the aforementioned strategies is specific for PPAR.

To avoid the potential side effects of PPAR agonists, strategies that regulate PPARα, such as the induction of PC could be of clinical interest. PC is an adaptive mechanism that consists of a brief period of I/R, resulting in marked

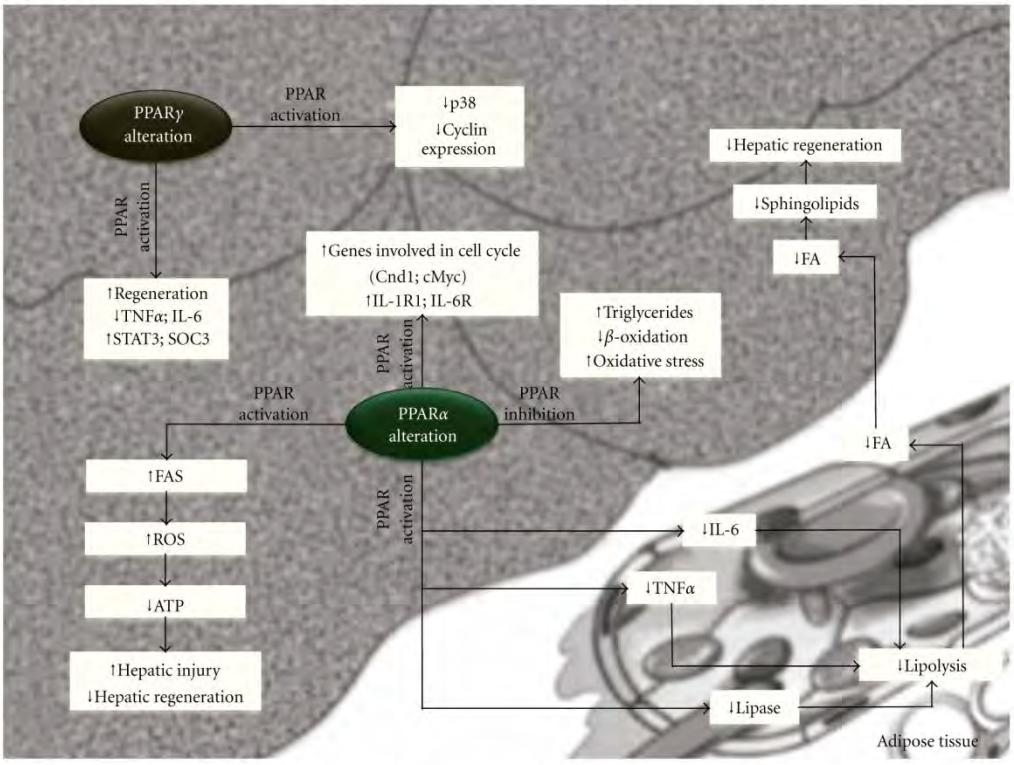

Figure 3: PPAR and hepatic regeneration. Adenosine triphosphate: ATP; fatty acid: FA; interleukin-1 receptor: IL-1R; interleukin-6: IL-6; interleukin-6 receptor: IL-6R; tumor necrosis factor-alpha: TNF-α; signal transducer and activator of transcription 3: STAT3; suppressor of cytokine signalling 3: SOC3; reactive oxygen species: ROS.

resistance in the liver, prior to a subsequent prolonged ischemic stress. Our successes regarding the efficacy of PC in nonsteatotic and steatotic livers undergoing warm ischemia (associated with PH) and liver transplantation [1, 2, 14, 91–93] have resulted in the clinical application of PC.

Several studies have demonstrated the effectiveness of PC in the resection of steatotic and nonsteatotic livers in clinical practice [94–96]. In such studies, the authors primarily performed liver resection via a continuous Pringle maneuver. However, other data indicate that PC does not improve postoperative liver function and does not affect morbidity or mortality after hepatectomy under vascular exclusion of the liver with the preservation of caval flow [97, 98]. The discrepancy between these differential effects of PC during hepatic resection might have arisen from the absence of back flow perfusion of the liver during vascular exclusion compared with that during the Pringle maneuver, which involves interruptions only to the inflow to the liver. In addition, the ischemic period used by Azoulay et al. [97] was longer (10 min on average) that that used by Clavien et al. [94]. All of these could explain, at least partially, the different effectiveness of PC in the clinical practice of liver surgery.

In the past decade, serious efforts have commenced to translate some of the robust benefits of PC against ischemia reperfusion to liver transplantation in clinical practice. It is fair to conclude that the overall clinical results have been less impressive than the observations in experimental animals. There are different data on the effectiveness of PC in I/R injury associated with liver transplantation [99–102]. However, these differential effects cannot be explained by the use of PC periods that have proved experimentally ineffective or by the clinical use of different cold ischemic times from those evaluated experimentally. However, the reduced proportion of subjects with steatosis enrolled in PC trials and the presence of brain death in clinical liver transplantation, which has thus far been evaluated in experimental studies of liver transplantation, should be considered.

As previously mentioned, the proportion of subjects with steatosis who have been enrolled in PC trials to date has been small (10%). Thus, in the future, clinical trials must make serious efforts to include a larger proportion of donor with steatotic livers to clarify the effectiveness of PC in liver transplantation in clinical practice. The benefits of PC are more likely to become clinically meaningful in patient

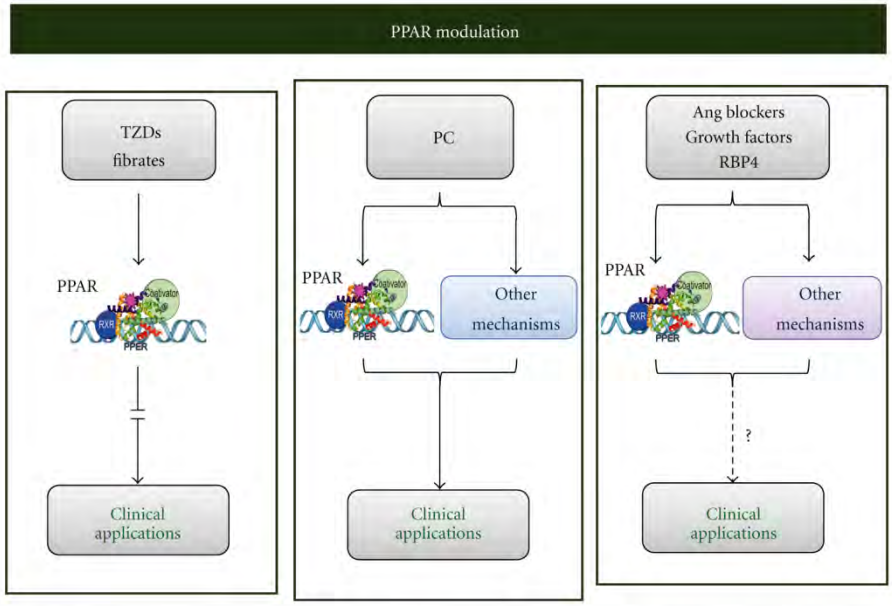

FIGURE 4: Clinical application of strategies that regulate PPARs. Angiotensin: Ang; peroxisome proliferator-activated receptors: PPARs; ischemic preconditioning: PC; retinol binding protein: RBP4; thiazolidinediones: TZDs.

groups with an increased risk of morbidity and mortality following PH, that is, in patients with hepatic steatosis and cirrhosis. In fact, in the largest prospective randomized study of PC in PH, Clavien et al. [94, 103] demostrated that PC was more effective in reducing reperfusion injury in patients with steatotic livers. Furthermore, Li et al. [104] reported that PC decreased the risk of hepatic insufficiency and shortened the hospital stay in patients with cirrhosis who underwent PH. There is the remote possibility that PC may not be effective in the context of brain death. Deceased organ donors have hemodynamic instability with decreased mean arterial pressure, portal venous, and hepatic tissue blood flow. Furthermore, brain death induces a multifaceted, intense systemic inflammatory response that is manifested in many organs, including the liver. It is very likely that such a framework of inflammatory response, well entrenched before the induction of PC, would interact with the various mechanistic aspects of PC and modulate the eventual PC response. To our knowledge, there are no studies of PC in the livers in brain-dead animals. Additional experimental studies of PC of the liver and other organs in brain-dead animals are needed to fill the knowledge gaps. The clinical observations suggest that PC alone may be insufficient to provide easily demonstrable clinical benefits in the presence of brain death. In that context, PC may be more effective when combined with physical, chemical, and pharmacological PC methods. Such experimental investigations could address an important clinical problem in liver transplantation, as more than 80% of livers used for transplantation are taken from cadaveric donors and approximately 20% of all brain-dead donors have a mild-to-moderate hepatic steatosis [105].

7. Conclusions and Perspectives

The use of experimental models has contributed to a better understanding of the multifaceted roles of PPARs. Strategies based on PPAR regulation have the potential to improve the postoperative outcomes of patients undergoing hepatic resections and to increase the number of organs suitable for transplantation, as these strategies may improve the outcomes of patients receiving marginal grafts that would not otherwise have been transplanted, leading to new possibilities for small steatotic liver transplants. Before a complete definition of a successful therapeutic strategy based on PPAR regulation is formed, several additional points need to be addressed. Comparative studies of the roles of different PPAR isoforms in hepatic I/R are required. We recently mapped the effects of PPAR on the pathways involved in the inflammatory process and lipid metabolism, and the effects of PPAR differ according the experimental model used. Therefore, therapeutic strategies targeting PPAR regulation also differ according to the surgical procedure. Moreover, the response of different types of liver to PPAR stimulation might differ and involve different signal transduction pathways that are at present marginally understood. Further research is required to select drugs that regulate PPAR with minimal side effects and optimize such potential treatments (e.g., dose and pharmacokinetics) before being translated into

treatments for human disease. Pharmacological strategies that specifically regulate PPAR including fibrates and TZDs might be inappropriate for clinical liver surgery due to their potential side effects. Conversely, surgical strategies such as PC have been applied in clinical surgery; however, these strategies do not exert their effects exclusively on PPAR, as they affect multiple aspects of I/R injury. Only a full appraisal of the role of PPAR in hepatic I/R and studies on the structure of this transcription factor will permit the design of new protective strategies for clinical liver surgery based on the specific regulation of PPAR without adverse effects.

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REVIEW ARTICLE

Current knowledge on oxidative stress in hepatic ischemia/reperfusion

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Abstract

Ischemia/reperfusion (I/R) injury associated with hepatic resections and liver transplantation remains a serious complication in clinical practice, despite several attempts to solve the problem. The redox balance, which is pivotal for normal function and integrity of tissues, is dysregulated during I/R, leading to an accumulation of reactive oxygen species (ROS). Formation of ROS and oxidant stress are the disease mechanisms most commonly invoked in hepatic I/R injury. The present review examines published results regarding possible sources of ROS and their effects in the context of I/R injury. We also review the effect of oxidative stress on marginal livers, which are more vulnerable to I/R-induced oxidative stress. Strategies to improve the viability of marginal livers could reduce the risk of dysfunction after surgery and increase the number of organs suitable for transplantation. The review also considers the therapeutic strategies developed in recent years to reduce the oxidative stress induced by hepatic I/R, and we seek to explain why some of them have not been applied clinically. New antioxidant strategies that have yielded promising results for hepatic I/R injury are discussed.

Keywords: liver, ischemia, reperfusion, steatosis, oxidative stress

Introduction

Reactive oxygen species (ROS) are largely generated from mitochondrial energy metabolism via oxidative phosphorylation in the respiratory chain. Because of the existence of antioxidant systems, the redox balance is well maintained. However, ischemia/reperfusion (I/R), an unavoidable process when performing hepatic resections and liver transplantation, increases the production of ROS from various sources and results in the disturbance of this delicate balance. The increase in ROS consumes endogenous antioxidants and induces the expression of antioxidant enzymes in order to maintain the redox balance. When the injury is pronounced or persistent, compensatory responses become inadequate to correct the imbalanced redox state, giving rise to oxidant stress and leading to inflammatory responses and hepatic damage. Formation of ROS and oxidant stress are the disease mechanisms most commonly invoked in hepatic I/R injury. The present review examines published results regarding possible mechanisms responsible for ROS and their effects in the context of hepatic I/R injury. Given that in the event of I/R-induced donor organ damage, oxidant stress depends on the donor conditions (steatotic, small-for-size, and aged livers) we also review the effect of oxidative stress on marginal livers, which have been shown to suffer increased oxidative injury from exposure to I/R, as compared with histologically normal livers. Our review of pharmacological and surgical strategies for regulating oxidative stress may help researchers to find effective strategies in the treatment of hepatic I/R.

Effects of oxidative stress in hepatic ischemia/reperfusion

Reperfusion phases in hepatic ischemia/reperfusion

Hepatic I/R injury occurs when there is a transient blockage of blood supply to the liver and subsequent reestablishment of the blood supply. The injury process is more extensive during the reperfusion period than during ischemia [1]. Growing evidence supports the notion that oxidant stress is the major initiator in eliciting signaling pathways that lead to the onset of necrosis/apoptosis during the hepatic I/R procedure, especially in the early stage of the process [2].

The destructive effects of I/R result from the acute generation of ROS subsequent to reoxygenation. These ROS inflict direct tissue damage and initiate a cascade of deleterious cellular responses leading to inflammation, cell death, and organ failure [3,4]. The development of hepatic I/R injury can be divided into initial and late phases [5]. The initial phase (< 2 h after reperfusion) is characterized by oxidative stress. Here, the excessive ROS cause tissue damage and cell death by binding and altering cellular

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macromolecules (including DNA, proteins, and lipids), thus affecting their function. The key event in the initial phase of reperfusion injury is activation of the macrophages that are the primary source of extracellular ROS, leading to endothelial injury and further release of proinflammatory cytokines [6–8]. The activation of Kupffer cells (KC) in the vasculature results in the generation of ROS, causing oxidative stress. CD4+ T-lymphocytes produce granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon gamma (INF-γ), which amplify KC activation. Reperfusion damage also appears to be related to ROS formation from xanthine oxidase (XOD). It is known that xanthine dehydrogenase (XDH) is metabolized to XOD during hypoxia, and upon reperfusion, oxygen reacts with XOD to produce ROS [2].

Tumor necrosis factor (TNF)-α is also of particular importance in I/R injury as it induces expression of adhesion molecules on vascular endothelial cells and stimulates chemokines, leading to the recruitment of neutrophils which release more ROS and proteases and create further injury [2]. Platelet-activating factor can prime neutrophils for ROS generation [2], whereas leukotriene B4 contributes to the amplification of the neutrophil response. Neutrophil priming and activation for ROS formation involve complement factors, damage-associated molecular patterns such as high-mobility group box 1 protein (HMGB1) and DNA fragments which enhance ROS priming [9]. In addition, I/R initiates protein misfolding in the endoplasmic reticulum (ER), which can activate a highly conserved unfolded protein response (UPR) signal transduction pathway. The UPR is characterized by coordinated activation of three ER transmembrane proteins, inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor (ATF)-6. If the damage is so severe that homeostasis cannot be restored, ER stress signal transduction pathways ultimately initiate ROS production, apoptosis, and necrosis.

Mitochondria are the main cell components responsible for events involving oxidative stress. In addition, oxidant stress is a potent trigger of mitochondrial permeability transition (MPT) pore opening in hepatocytes [2]. The mitochondrial membrane potential collapses when the MPT occurs, leading to failure of ATP synthesis, release of cytochrome *c*, and cell death [10,11]. It has been documented that release of lysosomal iron and its uptake into mitochondria can act synergistically with oxidant stress to promote the MPT and cell death [2]. Thus, damaged mitochondria produce progressively greater amounts of ROS, which, in turn, causes progressively greater damage to the very same mitochondria, thereby increasing the likelihood of reduced ATP production and compromised cell function [12]. In addition to the high ROS-generating systems, livers show low levels of antioxidants such as glutathione (GSH) and superoxide dismutase (SOD), thereby exacerbating the injurious effects of ROS. Alterations in the renin-angiotensin system (RAS), retinol binding protein 4 (RBP4), and peroxisome proliferator activated receptor gamma (PPRγ) also contribute to oxidative stress. In the late phase of injury, between 6 and 24 h after reperfusion, an evolving inflammatory process occurs that is mediated by oxidants of extrahepatic cellular origin [13]. This late phase of I/R injury is caused by neutrophil activation, ROS, TNF-α, and IL-1β, and it results in more substantial injury than was initially caused by KC. There is also upregulation and expression of inducible nitric oxide synthase (iNOS), creating large quantities of nitric oxide (NO) that results in further creation of reactive nitrogen species [14]. It is proposed that peroxynitrite ($ONOO^-$), the product of superoxide anion and NO by iNOS, may be the cause of the damage [2]. Peroxynitrite can cause lipid peroxidation, inhibition of the mitochondrial respiratory chain, inhibition of Na+/K+ ATPase, and/or oxidative protein modifications [2] (Figure 1).

Controversies regarding the mechanisms responsible for ROS production in hepatic I/R

We will know consider potential mechanisms responsible for oxidative stress during hepatic I/R, including xanthine/XOD, the activation of KC and neutrophils, and the imbalance between NO and superoxide anion production.

One main chemical source which has been shown to contribute significantly to overall pronounced oxidant stress during hepatic I/R is XO, which generates superoxide anions ($O2^-$) during the conversion of hypoxanthine to xanthine [2]. Note, however, that the injurious effects of ROS are not limited to the liver, since there is evidence indicating that the release of xanthine and XOD from the liver into the bloodstream plays an essential role in the pathogenesis of systemic complications of hepatic I/R, including neutrophil infiltration and oxidant stress into the lung. Experiments based on XOD inhibition with allopurinol suggest that the xanthine/XOD system is the main ROS generator in hepatocytes [2] and it has also been implicated in liver transplantation-related lung damage [13]. However, several studies suggest that XOD is of limited importance in clinical practice, since this enzyme exists as XDH and the conversion to the ROS-generating oxidase requires lengthy ischemic times. Accordingly, experimental data indicate that xanthine/XOD only plays a crucial role in hepatic I/R injury in conditions in which significant conversion of XDH to XOD occurs (80–90% of XOD), for example after 16 h of cold ischemia. By contrast, this ROS generation system does not appear to be crucial with shorter ischemic periods such as 6–8 h of cold ischemia, a period commonly used in clinical practice [13,14]. In addition, ROS formation from XOD depends on the xanthine and hypoxanthine substrates. Although these substrates accumulate during ischemia, they are metabolized relatively quickly and during perfusion they are flushed out from the liver together with other metabolites such as lactate and pyruvate. It is also important to consider the drugs used to inhibit xanthine/XOD, since allopurinol, for example, is not only a potent inhibitor of XOD but may also improve ischemia-induced mitochondrial dysfunction [2,15].

Under hepatic I/R conditions, mitochondrial dysfunction may impair electron flow and enhance superoxide

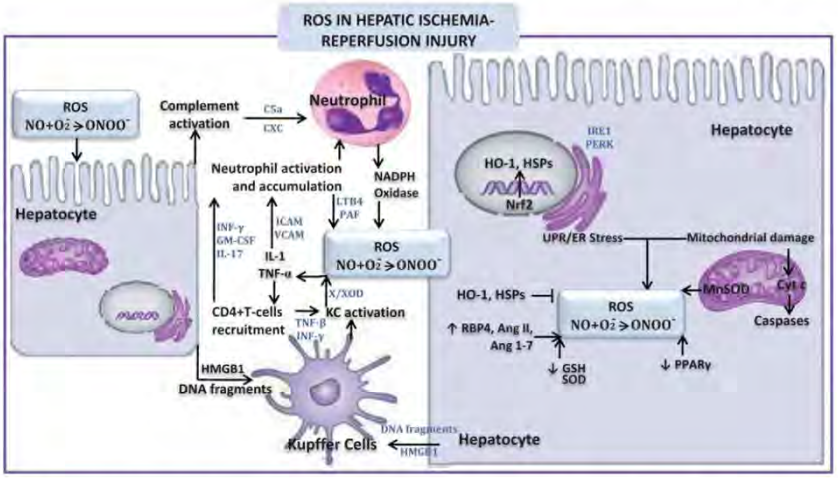

Figure 1. Mechanisms of ROS injury in hepatic ischemia/reperfusion. Ang, angiotensin; c5a, complement factor 5a; CXC, chemokines; Cyt c, cytochrome c; ER, endoplasmic reticulum; GM-CSF, granulocyte-macrophage colony-stimulating factor; GSH, glutathione; HMGB1, high-mobility group box 1 protein; HO-1, heme oxygenase-1; HSPs, heat-shock proteins; ICAM, intracellular cell adhesion molecule; IL, interleukin; INF, interferon; IRE1, inositol-requiring enzyme 1; LTB4, leukotriene B4; MnSOD, manganese superoxide dismutase; NADPH, nicotinamide adenine nucleotide phosphate; NO, nitric oxide; Nrf2, nuclear factor-erythroid 2 p45-related factor 2; $ONOO^-$, peroxynitrite; PAF, platelet-activating factor; PERK, PKR-like ER kinase; PPAR, peroxisome proliferator-activated receptor; RBP4, retinol binding protein 4; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, tumor necrosis factor; UPR, unfolded protein response; VCAM, vascular cell adhesion molecule; X/XOD, xanthine/xanthine oxidase.

formation. Indeed, mitochondria can be a primary target during the very early injury phase, and may also be a target of the inflammatory response at later times. However, some data challenge the pathophysiological relevance of intracellular oxidant stress during reperfusion [16,17]. For instance, Grattagliano et al. [17] demonstrated that mitochondria do not seem to participate actively in reperfusion-induced oxidative stress. Thus, in order to clarify the importance of mitochondria as a source of ROS, one needs to take into account the different experimental models evaluated, including ischemia times. In this regard, it has been shown that prolonged ischemia times are required to trigger mitochondrial oxidant stress in the liver.

Some studies under cold ischemia conditions implicate KC in ROS production. The conversion from XDH to XOD following cold storage is very slow in endothelial cells and hepatocytes, but much faster and greater in KC. These macrophages are the main source of vascular oxidant stress during the early reperfusion phase after both warm [18] and cold ischemia. In addition, some studies have shown that increased vascular oxidant stress under ischemia conditions was attenuated by inactivation of KC but not by a high dose of allopurinol [19]. Although ischemic stress can trigger an oxidant burst in macrophages during reoxygenation [20], the prolonged ROS formation during the early phase of reperfusion *in vivo* is, at least in part, triggered by activated complement fragments [2]. These resident phagocytes also produce proinflammatory mediators, such as interleukins and TNF-α, enhancing the expression of endothelial cell adhesion molecules and priming circulatory neutrophils [21,22]. In line with this, treatment with gadolinium chloride or complement depletion [2] has been shown to reduce the capacity of KC to generate ROS, thus protecting against hepatic I/R injury [2]. On the other hand, there is a range of potentially conflicting results with regard to the role of KC in ROS generation associated with hepatic I/R, since the elimination of KC was found not to modify the deleterious effects of I/R [2,23,24]. To explain this, it should be noted that the stimulatory state of KC after I/R depends on the duration of ischemia, and it may also differ between warm and cold ischemia, which probably leads to different developmental mechanisms of liver damage.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the main source of superoxide formation by neutrophils [25,26]. The primary ROS generated by neutrophils include $O2^-$, HO^-, H_2O_2, and their derivatives, such as $ONOO^-$. Activated neutrophils also release elastase, cathepsin G, heparanase, collagenase, and hydrolytic enzymes that appear to be directly cytotoxic to hepatocytes [27,28]. Neutrophil recruitment into the liver starts during the first few hours after initiation of reperfusion. However, at that time, these neutrophils are only primed for enhanced ROS formation [26], and neutrophil-mediated oxidant stress actually is observed at 6–24 h of reperfusion [29], correlating with a neutrophil-mediated

injury phase. The main cytotoxic ROS, which are generated during a neutrophil attack and actually end up inside a hepatocyte, are hydrogen peroxide and hypochlorous acid [25,29].

The imbalance between NO and superoxide anion production under hepatic I/R represents another mechanism responsible for oxidative stress. Under normal physiological conditions, both NO and superoxide anion are produced by endothelial cells, with NO production exceeding superoxide anion generation by two to three orders of magnitude. This allows NO to (i) effectively scavenge intracellular superoxide anion, (ii) prevent platelet aggregation, and (iii) minimize adhesive interactions between neutrophils and the endothelial cell surface [30]. However, following hepatic I/R the formation of superoxide anion exceeds that of NO because of the overproduction of superoxide anion and/or the dramatically reduced bioavailability of NO due to (i) low intracellular levels of cofactors for the synthesis of NO (NADPH and oxygen), (ii) L-arginine breakdown by the large amount of arginase released after ischemic insult, (iii) inhibition of endothelial cell NO synthase activity, and (iv) rapid inactivation of NO by superoxide anion. Thus, under these conditions the beneficial actions of NO are lost [30,31].

It is well known that excessive levels of iNOS-derived NO production may have a detrimental effect through the generation of NOS-derived superoxide or $ONOO^-$ [32,33]. $ONOO^-$ leads to depletion of cellular antioxidant defenses, inactivation of enzymes, and nitration of tyrosine residues in proteins that may adversely affect their function, and also signal transduction processes [34,35]. $ONOO^-$ is a predominant form of ROS in steatotic liver grafts, and the injurious effects of exogenous NO donors in relation to hepatic injury and oxidative stress in steatotic grafts have been shown to be associated with increased nitrotyrosine levels. This could explain, at least partially, the increased vulnerability of steatotic grafts to oxidative stress, as well as the injurious effects of exogenous NO on steatotic liver transplantation. Interestingly, however, exogenous NO has been shown to protect non-steatotic livers against hepatic I/R injury. It should also be noted that much less ROS is produced in nonsteatotic than in steatotic livers. To understand these effects, one needs to consider the fact that $ONOO^-$ forms under conditions of high production of both NO and ROS [34–38].

Oxidative stress in marginal livers undergoing I/R

Steatotic livers

Several studies indicate that an increased sensitivity of fatty hepatocytes to the injurious effects of ROS could explain the poor tolerance of steatotic livers to I/R [36–38]. Steatotic livers are more susceptible than nonsteatotic livers to lipid peroxidation because of either lower antioxidant defenses or their greater production of ROS or both [39]. Mitochondrial ROS generation dramatically increases during reperfusion and mitochondrial structures are exposed to attack by the ROS generated both outside and inside these organelles. This leads eventually to the dysfunction of important mitochondrial processes including those responsible for ATP synthesis. Steatotic livers are known to synthesize less ATP than do nonsteatotic livers during postischemic reperfusion [40]. One of the important effects of uncontrolled production of ROS is the peroxidation of membrane and other cellular lipids, resulting in structural alterations of membranes and functional impairment of cellular components [41,42]. A number of studies have focused on preventing the increased oxidative stress observed in steatotic livers [37,41,43]. One of the problems, however, it that both dietary high fat and alcohol exposure have been found to produce superoxide dismutase (SOD)/catalase-insensitive ROS, which may be involved in the mechanism of failure of steatotic livers after hepatic I/R [37,41,43].

Aged livers

Age appears to influence the sensitivity of the liver to oxidative stress. One recent study reported that NO plays an important role in oxidative stress in the liver of older rats subjected to *ex vivo* reperfusion. The extent of cellular damage was related to a balance between the production of oxidants and their removal by the antioxidant system [44]. Under warm hepatic ischemia, mature adult mice had a greatly increased neutrophil function, increased intracellular oxidants, and decreased mitochondrial function compared with young adult mice. A vicious cycle may occur in which damaged mitochondria produce progressively greater amounts of ROS, leading, in turn, to progressively greater damage to mitochondrial, cytosolic, and nuclear compartments, before finally resulting in dysfunctional or defective mitochondria. This is the basis of the mitochondrial theory of aging. In addition, mature adult mice had much lower hepatic expression of a cytoprotective protein, heat shock protein (HSP) 70 (HSP70), than did young adult mice. By contrast, serum HSP70 levels, which have been linked to subsequent tissue injury, were higher in mature adult mice than in young adult mice. All of these alterations contributed to the increased liver injury after I/R in mature adult mice compared with young adult mice.

Small-for-size liver grafts

In recent years, research on small-for-size syndrome after transplantation has been more focused on the extensive preformation of the recipients of living donor liver transplantation (LDLT) [1]. The major concern with this surgical procedure is graft-size disparity. Liver hepatectomy requires posterior regeneration to restore the liver/body ratio. However, there is evidence that small-for-size liver grafts are more vulnerable to I/R injury after transplantation than are standard-size liver grafts [45]. ROS originating after reperfusion are known to induce DNA damage and inhibit cell division after

hepatectomy [46]. Furthermore, previous results indicated that inhibition of KC with gadolinium reduced oxidative stress following reduced-size liver transplantation, suggesting that these macrophages might be a potential ROS source under these surgical conditions. In reduced-size liver grafts, XDH was converted to the oxygen radical-producing form XOD as a consequence of cold ischemia. Xanthine levels increased after ischemia and remained high after reperfusion. However, the potential involvement of xanthine/XOD in the oxidative damage observed in reduced-size liver graft was not investigated in that study [46]. Previous data in living-related liver transplantation suggest that strategies aimed at increasing HO-1 might reduce oxidative stress and protect against damage. HO-1, a rate-limiting step in the conversion of heme, is believed to play a key role in the maintenance of antioxidant and oxidant homeostasis during times of cellular injury and stress [47].

Antioxidant strategies against hepatic ischemia/reperfusion injury

Preconditioning

Perhaps the most common investigational method of reducing I/R injury is traditional ischemic preconditioning (PC), induced by a brief period of I/R before subsequent prolonged hepatic ischemia [48] (Figure 2). The benefits of ischemic PC in relation to oxidative stress could be explained by the induction of antioxidants such as SOD, NO, and HSPs, as well as by its effect on XDH/XOD [2,39,49]. PC, through NO, reduced the accumulation of xanthine during ischemia and prevented the conversion of XDH to XOD, thus preventing the deleterious effect of this ROS generating system on liver [13,50]. The benefits of ischemic PC with regard to mitochondrial dysfunction and neutrophil and KC activation in hepatic I/R have been reported to be associated with reduced oxidative stress. Ischemic PC, mediated by adenosine, regulated the imbalance between pro- and anti-inflammatory cytokines, thus reducing ROS production in steatotic livers undergoing I/R. Ischemic PC, through the peroxisome proliferator-activated receptor ($PPAR_{\alpha}$) inhibits adiponectin accumulation in steatotic livers and adiponectin-worsening effects on oxidative stress and hepatic injury in hepatic resections. In addition to the effect of ischemic PC on oxidative stress, it should be noted that this surgical strategy involves a substantial number of mechanisms that promote cellular resistance to stress.

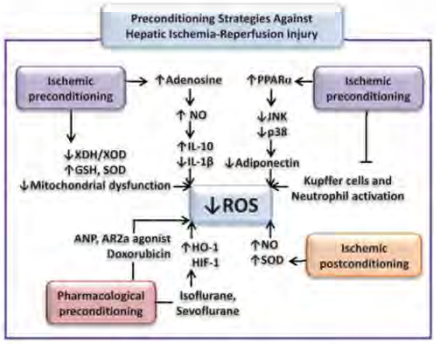

Figure 2. Preconditioning strategies against hepatic ischemia/reperfusion injury. AR2, adenosine receptor agonist, adenosine type II receptor agonist; ANP, atrial natriuretic peptide; GSH, glutathione; HIF-1, hypoxia-inducible factor; HO-1, heme oxygenase-1; IL, interleukin; JNK, Jun-Nterminal kinase; NO, nitric oxide; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SOD, superoxide dismutase; XDH/XOD, xanthine/xanthine oxidase.

Numerous attempts have been made to reproduce the benefits of ischemic PC pharmacologically to avoid invasive measures and provide more persistent treatment postsurgery. By modulating intracellular or extracellular components known to be part of the protective pathway, it is possible to simulate the preconditioning effect. This can be achieved by providing pharmacological stimulation before I/R with an adenosine receptor 2 agonist [51] or with other chemical agents such as doxorubicin or atrial natriuretic peptide [52]. Some of the effects of pharmacological PC involve improved antioxidant capacity [53]. However, their possible clinical application seems limited owing to the difficulties in implementing them in clinical practice. Furthermore, toxicity problems and side effects have been identified.

Volatile anesthetics as pharmacological PC agents have been investigated for their capacity to protect against hepatic I/R injury. Isoflurane can protect against I/R injury at clinically relevant levels [54], and sevoflurane has been shown to reduce hepatic injury and improve outcome in clinical patients postsurgically, with an even better outcome in steatotic patients [55]. It appears that anesthesia also induces both hemeoxygenase-1 (HO-1) [54] and hypoxia-inducible factor (HIF)-1 [56], so the effect may be mediated through the antioxidant activity oh these enzymes.

Ischemic PC has been successfully applied in human liver resections in both steatotic and nonsteatotic livers. Although some clinical studies showed benefits of ischemic PC in liver transplantation, further randomized clinical studies are necessary to confirm whether ischemic PC is indeed appropriate in this context. Ischemic PC did reduce the lipid peroxidation observed in fatty livers after hepatic reperfusion [2,39]. In addition, the benefits of PC with regard to oxidative stress, specifically in relation to the ROS generating system xanthine/XOD, have been documented in reduced-size liver transplantation [46]. This endogenous protective mechanism would probably be more effective against ROS than are pharmacological strategies based on the administration of antioxidants, which in order to be effective need to reach the site of action in adequate concentrations. Ischemic PC also prevented GSH depletion during hepatic ischemia, and this endogenous protective mechanism was more

effective against ROS and hepatic damage than was GSH–ester pretreatment [41]. Although few studies on the effects of ischemic postconditioning in hepatic I/R have been conducted to date, this surgical strategy, based on brief periods of I/R after sustained ischemia, might protect against oxidative stress damage throughout NO generation and SOD overexpression [57] (Table I).

Pharmacological therapy

This section of the review considers prosurvival genes and antioxidant drugs with antioxidant properties. Different endogenous genes or gene products have been established as highly protective when induced before and, in some cases, after the start of ischemic injury. For their part, antioxidant enzymes play a fundamental role in maintaining the delicate redox balance and are essential for preserving physiological function and coping with oxidant stress from endogenous or exogenous sources. The gene expression of most antioxidant enzymes such as SOD, glutathione peroxidase (Gpx), catalase, or HO-1 is inducible under inflammation or other stressful conditions, and this induction represents the key mechanism for the body in response to a variety of stressors. Generally speaking, all these gene components are in some way involved in direct scavenging of ROS or general detoxifying enzymes capable of removing endogenous threats. Many of these genes are controlled by the transcription factor nuclear *factor erythroid 2 p45-related factor* 2 (Nrf2)–*Kelch-like ECHassociated protein 1* (Keap1) system [58,59]. Under conditions such as oxidative stress or hypoxia, Nrf2 is activated and its translocation to the nucleus alters gene expression, including that of HO-1 and GSH [60], which could be potential therapeutic targets to combat I/R-induced oxidant stress [58]. The results found in hepatic I/R indicate a disturbance in the pro-oxidant/antioxidant balance, in favor of the pro-oxidant species. Specifically, the decrease in SOD activity and GSH levels was accompanied by an increase in H_2O_2 levels. Several hypotheses have been suggested to explain the weakening of antioxidant defense mechanisms during the I/R process [61]. The drop in GSH levels may result either from an arrest of synthesis or a higher consumption. The decrease in SOD activity levels could be associated with their inhibition or inactivation as part of the I/R process, or with free radical attack itself. It is known that maintaining or strengthening endogenous antioxidant mechanisms may attenuate the injurious effect of ROS in the I/R process [2], and in order to achieve this, the administration of antioxidants should be considered. The following are common antioxidant enzymes chosen to prevent or treat hepatic ischemic conditions.

SOD, which catalyzes the dismutation of $O2^-$ to H_2O_2, is a major ROS scavenger. There are three isozymes of SOD, each displaying unique subcellular locations and playing antioxidative roles in various compartments [2]. Cu/Zn-SOD is localized in the cytosol and nucleus of all cell types, and functions as the intracellular antioxidative system. Manganese SOD (Mn-SOD) is exclusively localized in the mitochondria [62]. The unique extracellular distribution and secretory nature of extracellular SOD (EC-SOD) enable it to offer antioxidative protection against ROS not only in the cytosol but also in the extracellular space. This is of interest because $O2^-$ present in the extracellular space cannot cross the cell membrane to be removed by intracellular SOD [63]. Some studies have reported that treatment with SOD reduce oxidative stress under hepatic I/R [64–70], although other studies showed no effect [2]. This is probably due to the difficulty of getting SOD into cells (Table I).

Catalase, a potent scavenger of H_2O_2, prevents the formation of HO^- when SOD is insufficient to remove $O2^-$ overload. Insufficient catalase activity can lead to an accumulation of H_2O_2, which may in turn be converted into more toxic HO^- in the presence of $Cu2^+$ and $Fe2^+$ or $O2^-$ [71,72]. Thus, catalase administration could result in additional antioxidative activity against oxidative stress. In a study carried by He et al., the combination of SOD and catalase gene delivery achieved additive effects in reducing oxidative stress in hepatic I/R. Note, however, that in some cases even intravenous administration of high doses of catalase or SOD resulted in only partial protection [67], largely due to poor bioavailability of the enzyme. As the half-life of SOD is only 6 min [73], gene delivery of SOD or catalase or their combination, may offer better protection against ROS overload. Indeed, one example of antioxidant gene therapy involves the suppression of ROS burst through SOD and catalase transfection by either adenovirus or liposomes or polyethyleneglycol [1,74,75]. SOD or catalase gene delivery mediated by liposomes led to an approximately 10-fold increase in liver SOD and catalase activity, and to a 50-fold increase in SOD mRNA levels. The elevated SOD or catalase activity in the liver-protected mice from hepatic I/R injury [65]. In line with this, gene delivery of cupric SOD1 and manganese SOD2 both reduced I/R injury as did gene delivery of SOD3 when given intramuscularly [76,77]. Transgenic mice overexpressing SOD1 were also protected against I/R injury [78]. Advances in antioxidant gene therapy will provide new opportunities to reduce the oxidative stress induced by hepatic I/R injury. However, there are a number of problems inherent in gene therapy, for example, vector toxicity, difficulties in increasing transfection efficiencies and protein expression at the appropriate time and site, and the problem of obtaining adequate mutants. Thus, further studies are needed before these gene therapy strategies based on inhibiting oxidative stress damage can be applied in clinical practice.

GSH, a highly effective antioxidant present in high concentrations in hepatocytes [140] is the Gpx substrate for H_2O_2 breakdown [80]. N-acetylcysteine (NAC) is the precursor to GSH and can be administered with effective uptake, although the effects of NAC on hepatic I/R injury are unclear [80–85]. In *in vivo* studies, treatment with NAC increased the levels of intracellular GSH, which reacts spontaneously with ROS or can be used for the

Table I. Effects of antioxidant strategies on hepatic I/R injury.

Drug	Animal	Experimental model	Effects
Adiponectin siRNA [138]	Steatotic rat	Warm I/R	↓ MDA and hepatic injury
Allopurinol (xanthine oxidase inhibitor) [142]	Mice	Warm I/R	↑ GSH ↓ xanthine oxidase, MDA and superoxide anion
Allopurinol [92,142]	Rat	Cold and Warm I/R	↓ ROS and hepatic injury
Ang II [141]	Steatotic rat	Warm I/R + PH	↓ MDA and nitrotyrosines
Ang-(1-7) [137]	Steatotic rat	Cold I/R, LT	↓ MDA, nitrotyrosines and cNOS
Apocynin (NADPH oxidase inhibitor) [142]	Mice	Warm I/R	↑ GSH ↓ NADPH oxidase, MDA and superoxide anion
Bucillamine [152]	Rat	Cold I/R	↑ GSH ↓ lipid peroxidation and hepatic injury
Catalase [72]	Rat	Warm I/R	↓ H_2O_2 and hepatic injury
Cobalt protoporphyrin (CoPP) (HO-1 inducer) [99]	Steatotic rat	*Ex vivo* cold I/R	↑ HO-1 ↓ hepatic injury
Coenzyme Q10 [92]	Rat	Warm I/R	= ROS
Desferrioxamine [149]	Dog	Cold I/R, LT	↓ hepatic injury
Grape seed extract [120]	Rat	Warm I/R	↑ GSH ↓ MDA and MPO
Green tea extract [121,125]	Rat	Warm I/R	↑ SOD ↓ MPO, ROS and hepatic injury
GSH [73,90–92]	Rat	Cold and Warm I/R	↓ ROS and hepatic injury
Idebenone [148]	Pig	Cold I/R	↓ lipid peroxidation and hepatic injury
IL-1 and IL-10 combination [139]	Steatotic rat	Warm I/R	↓ NO and MDA
Lipolic acid [150]	Rat	I/R, isolated rat liver perfusion	↓ hepatic injury
Melatonin [83]	Rat	Warm I/R	Mitochondrial redox status preservation ↓ MDA, iNOS, NO and hepatic injury
NAC [79,80]	Human	Cold I/R, LT	↓ αGST and hepatic injury
NAC [83]	Rat	I/R	↓ MDA and hepatic injury
ONO-1714 [14]	Pig	Warm I/R	↓ peroxynitrites and necrosis
Ozone [143]	Rat	Warm partial I/R	↓ ROS and Xanthine/XOD
PBA [140]	Steatotic rat	Warm I/R + PH	↓ MPO and MDA
PEG (Prostaglandin E1) [87]	Human children	Cold I/R, LT	↓ hepatic injury
Pentoxifylline and Coenzyme Q10 combination [147]	Rat	Warm I/R	↑ GSH ↓ MDA
Quercetin [123]	Rat	Warm I/R	↑ SOD and GSH ↓ ROS and MDA
SOD [92]	Rat	Warm I/R	↓ ROS and hepatic injury
SQ22536 (cAMP inhibitor) [136]	Steatotic rat	Cold I/R, LT	↑ GSH ↓ XOD
Trans-resveratrol [118,119]	Rat	Warm I/R	↑ Catalase, GSH and SOD ↓ MDA and hepatic injury
Tetrandine [122]	Mice	Warm I/R	↑ SOD ↓ MDA and neutrophil accumulation
α-tocopherol [37,108]	Rat	Cold and Warm I/R	↑ Catalase and GSH ↓ lipid peroxidation
Trimetazidine [151]	Rat	Warm I/R	↓ ROS and hepatic injury
TUDCA [154]	Steatotic rat	Warm I/R + PH	↓ MPO and MDA
Gene (vector)	**Animal**	**Experimental model**	**Effects**
Catalase (Poliplexes) [68]	Mice	Warm I/R	↑ SOD and GSH ↓ MDA and neutrophil accumulation
HO-1 (Adenovirus) [98]	Steatotic rat	*Ex vivo* cold I/R	↑ HO-1 ↓ hepatic injury
Cu/Zn-SOD (Adenovirus) [76]	Rat	Cold I/R, LT	↓ hepatic injury
Cu/Zn-SOD (Adenovirus) [75]	Mice	Warm I/R	↑ SOD ↓ lipid-derived ROS and hepatic injury
EC-SOD (Poliplexes) [68]	Mice	Warm I/R	↑ SOD, GSH ↓ MDA and neutrophil accumulation
SOD (Polyethylene glycol) [68]	Rat	Warm I/R	↓ MDA
SOD (Transgenic) [77]	Mice	Warm I/R	↑ SOD ↓ hepatic injury

Ang, angiotensin; I/R, ischemia/reperfusion; Cu/Zn.SOD, cytosolic SOD; EC-SOD, extracellular SOD; GSH, glutathione; GST, glutathione s-transferase; HO-1, heme oxygenase 1; LT, liver transplantation; MDA, malondialdehyde; MPO, myeloperoxidase; NAC, N-acetylcysteine; NO, nitric oxide; TUDCA, tauroursodeoxycholic acid; PBA, 4-phenyl butyric acid; PH, partial hepatectomy; ROS, reactive oxygen species; siRNA, small interference RNA; SOD, superoxide dismutase; XOD, xanthine oxidase.

detoxification of ROS by Gpx [86,87]. However, other experimental studies in steatotic livers, induced by a choline/methionine-deficient diet, show that NAC administration may help to restore hepatocellular integrity in the steatotic liver but without scavenging free radicals. Clinical trials of intravenous NAC have also produced equivocal results, although three studies did document improved liver function, decreased liver injury, lessened graft dysfunction, lessened rejection, and/or shortened hospitalization [88–90]. Clinical trials on the use of NAC as a protective agent during partial hepatectomy have now reached Phase IV. Although some of these trials have reported a reduction in hepatic injury parameters, there is no strong evidence in support of improved clinical outcome for these patients [8] (Table I).

As GSH is continuously released from hepatocytes into the vascular space, it can detoxify ROS generated by KC. Consequently, GSH administration has been shown to protect effectively against vascular oxidant stress during reperfusion after warm or cold ischemia [73,86,91,92]. However, this contrast with the results of some initial studies in this area that failed to show any protective effects of GSH in the setting of warm ischemia [93], a finding which may be due to limited cellular uptake of the large molecule. Gene transfer is an especially attractive option for GSH management, as GSH has a very short half-life in humans *in vivo* [94]. Studies have shown that the gene transfer of GSH synthesis components including glutamine cysteine ligase catalytic subunit (gclc), glutamine cysteine regulatory subunit (gclm), and GSH synthase offered protection against I/R injury by increasing intracellular GSH levels [59,60].

The stress response gene HO-1 can be induced by ischemic injury [95]. HO-1, a form of inducible HSP, is an enzyme that uses NADPH as a cofactor to catalyze the oxidative degradation of heme to biliverdin, carbon monoxide, and iron [47,96]. In this process it scavenges ROS and mitigates hepatic I/R injury [6,97–100]. Amersi et al. [101] showed that upregulation of HO-1 using cobalt protoporphyrin or adenovirus HO-1 protected against I/R injury in fatty livers of the Zucker rat. The mechanism by which HO-1 protects is thought to involve the formation of bilirubin and biliverdin, both of which have antioxidant activity. However, carbon monoxide, another byproduct of HO-1 enzymatic activity, can induce p38, a key component of ischemic protection [101]. Previous data indicated that HO-1 activators such as cobalt(III) protoporphyrin IX might protect both steatotic and nonsteatotic livers against warm I/R injury. Interestingly, a lower dose of HO-1 activator was required to achieve effective protection in steatotic livers, which under I/R showed higher HO-1 levels than did nonsteatotic livers [102]. Thus, although HO-1 induction appears to be one of the most promising therapeutic approaches in hepatic I/R models [103], the effective dose of HO-1 activators may depend on the liver type.

α-Tocopherol is a vitamin E analog that is preferentially absorbed in humans [104]. Consequently, it may also provide a first line of defense against DNA oxidative damage, acting mainly as a chain-breaking antioxidant in relation to lipid peroxidation of unsaturated fatty acids [105–108]. Pretreatment with α-tocopherol has been shown to attenuate reperfusion injury and lipid peroxidation and increase ATP synthesis, suggesting that it improves mitochondrial function [37,109–113]. α-tocopherol analogs also show beneficial effects, as does the combination of α-tocopherol with pentoxifylline [114,115]. Some research suggest that a combination of various antioxidants composed of vitamin E, taurine, and GSH may be more effective against hepatic I/R injury than would a single one [116]. β-carotene is another essential antioxidant found in the normal mammalian diet, and oral supplementation with this compound is well-tolerated. Like α-tocopherol, β-carotene can provides a first line of defense against DNA oxidative damage, and also acts mainly as a chain-breaking antioxidant in relation to lipid peroxidation of unsaturated fatty acids in the cell membrane [105,108]. Vitamin C, also known as ascorbic acid, is synthesized from l-gulono-γ-lactone and oxygen, thus creating l-ascorbate and hydrogen peroxide. Although this reaction occurs naturally in some animals, humans are not able to synthesize ascorbate, so intake is diet-dependent. In addition to regular dietary intake, low doses of vitamin C supplementation decrease liver peroxidase activity, while high-dose vitamin C has been shown to increase hepatic I/R injury, perhaps due to the excess reduction of iron [117]. One clinical study demonstrated improved prothrombin time and decreased complication in patients supplemented with an antioxidant vitamin infusion containing α-tocopherol acetate and ascorbate after liver resection [118] (Table I).

Natural antioxidants have been reported to be effective in reducing oxidative stress associated with hepatic I/R. Trans-resveratrol protected against hepatic I/R injury [119,120] through maintenance of GSH levels and the induction of SOD and catalase enzymes. Green tea catechins (GTCs) have been shown to be protective via maintenance of Mn-SOD and suppression of inflammation [121,122]. Tetrandrine, a traditional Chinese medicine, reduced oxidant stress, maintained SOD activity, and reduced the inflammatory response in hepatic I/R [123]. Quercetin is a naturally occurring flavonoid molecule that may also be protective against I/R injury [124]. Other antioxidant agents that have been shown to have beneficial effects in liver I/R injury include plant extracts such as cyaniding [125,126]. Although these natural compounds do appear to be beneficial in reducing oxidative stress damage in experimental hepatic I/R models, they generally require larger doses and prolonged pretreatment in order to be effective. There are obvious difficulties concerning the feasibility of long-term natural antioxidant administration in some I/R processes, particularly in the case of liver transplantation from a cadaveric donor, an emergency procedure in which there is very little time to pretreat the donor with these agents. Moreover, the potential side effects of these natural antioxidants should not be ruled out.

Antioxidants also play a role in a number of strategies designed to reduce mitochondrial and extracellular ROS formation, and which have been shown to be an effective treatment against MPT formation and subsequent hepatic I/R injury [2,127–130]. Administration of the antioxidants melatonin or edaravone led to some preservation of mitochondrial respiration potential and a reduction in mitochondrial swelling when given before I/R surgery [131,132,133]. However, the authors of these papers failed to measure mitochondrial membrane potential. When melatonin was given to a small cohort of patients after liver resection, they showed enhanced neutrophil apoptosis and neutrophil responsiveness [111]. These are promising results, but they are derived from small studies that need to be corroborated by larger clinical trials involving measures of clinical outcomes before any firm conclusions can be drawn (Table I).

To conclude this section, we will review a number of drugs that indirectly regulate oxidative stress in hepatic I/R. Adenosine, considered being one of the most likely candidates for mediating PC, has several beneficial biological actions in hepatic I/R processes, such as suppression of ROS [134,135]. Recent studies have also indicated that cAMP-blocking strategies including the use of SQ22536 protect against injury in steatotic liver grafts. This adenylate cyclase inhibitor reduced XOD activity, preserved antioxidant systems such as GSH, and protected against endothelial cell damage and microvascular disorders in steatotic liver grafts [136]. The benefits of Ang-(1-7)-Mas antagonists on oxidative stress in steatotic liver grafts have also been reported [137]. Previous studies in warm ischemia conditions indicated that the administration of adiponectin siRNA protected against the vulnerability of steatotic livers to oxidative stress [138]. Similar results were observed when recombinant IL-10, anti-IL-1 antibodies, or ER inhibitors were administered in steatotic livers undergoing I/R [139,140]. The benefits of Ang II receptor antagonists on $ONOO^-$ production have been observed in steatotic livers undergoing partial hepatectomy under I/R [141]. In addition, research has shown that interventions targeting CD11b or CD18 and NADPH oxidase effectively suppressed neutrophil-induced oxidant stress and liver injury during hepatic I/R [25,142]. NO derived from cNOS as well as iNOS inhibitors are also effective in reducing the oxidant stress and liver injury [2,39]. Treatment with ozone prevented the deleterious effects of XOD-derived ROS from canceling out the protection conferred by adenosine on hepatic I/R injury [143]. The overexpression of dominant-negative Rho kinase interrupted the function of native Rho-kinase activity and downstream Nox activation in hepatocytes, reduced ROS production, suppressed the release of pro-inflammatory cytokines, and ameliorated lethal liver injury with a significant prolongation of survival in liver transplantation [144]. Other agents with antioxidant properties in liver I/R injury include prostaglandin E2 [145,146], coenzyme Q/pentoxifylline [147], idebenone [148], desferrioxamine [149], lipoic acid [150], trimetazidine [151], bucillamine [152], and aminoguanidine [153] (Table I).

Future perspectives of antioxidant therapy

A large number of intervention strategies have shown a correlation between ROS inhibition and protection, but they do not clearly establish whether preventing ROS formation or scavenging ROS is the main cause of the reduced injury. Moreover, the conflicting results regarding the mechanisms responsible for oxidative stress make it difficult to develop potential strategies for inhibiting oxidative stress in clinical practice. The response of antioxidant systems also depends on surgical conditions, including ischemia times. In addition, the response of different types of livers to antioxidant strategies might differ and may involve different signal transduction pathways that are at present only marginally understood. More and improved antioxidant strategies therefore need to be developed and mechanistically tested. In this regard, therapeutic interventions that target multiple antioxidant pathways, such as ischemic PC, may be a more promising strategy than attempting to improve the antioxidant capacity of cells that act as free-radical scavengers. Furthermore, ischemic PC avoids the potential side effects derived from drug administration. Indeed, the possible side effects of some drugs can often limit their use in human liver surgery associated with hepatic resection or liver transplantation. Moreover, it is important to consider the difficulties which derive from pharmacological treatment. For instance, SOD and GSH show inadequate delivery to intracellular sites of ROS action, and no study has yet addressed the question of whether an antioxidant gene can be delivered during the window of donor organ procurement and implantation, or after grafts are implanted. The delivery of an antioxidant gene during the transplant window or posttransplant would be helpful for addressing not only oxidant stress during the reperfusion period but also graft failure after transplant. However, neither viral nor non-vector systems are fully optimized for clinical use yet. More studies of gene therapy are therefore needed so as to critically analyze the best administration routes, doses, and time points of liposome delivery, as well as to evaluate efficacy, species differences in immune responses, and adverse effects in large animals. Whether or not the gene therapy approach can be translated into clinical practice rests on the success of such research. Finally, strategies aimed at inhibiting effectively oxidative stress in hepatic I/R injury could reduce damage and increase liver regeneration in surgical procedures including hepatic resections and reduced-size liver transplantation. In addition, these antioxidant strategies might increase the number of organs suitable for transplantation since they may improve outcomes for marginal grafts that otherwise would not be transplanted.

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Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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2

Ischemia-Reperfusion Injury Associated with Liver Transplantation in 2011: Past and Future

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1. Introduction

Liver transplantation has evolved as the therapy of choice for patients with end-stage liver disease. However, the waiting list for liver transplantation is growing at a fast pace, whereas the number of available organs is not growing at a proportional rate. The potential use of steatotic livers for transplant, one of the most common types of organs from marginal donors, has become a major focus of investigations. However the clinical problem is still unresolved since steatotic livers are more susceptible to ischemia-reperfusion (I/R) injury and, when used, have poorer outcome than non-steatotic livers. Indeed, the use of steatotic livers for transplantation is associated with increased risk of primary non-function or dysfunction after surgery. Therefore, minimizing the adverse effects of I/R injury could improve outcomes in steatotic liver surgery, increase the number both of suitable transplantation grafts and of patients who successfully recover from liver transplantation.

The present review focuses on the complexity of hepatic I/R injury, summarizing conflicting results obtained from the literature about the mechanisms responsible for it. We also review the therapeutic strategies designed in past years to reduce I/R injury, attempting to explain why most of them have not been applied clinically. Finally, we will consider new potential protective strategies that have shown promising results for I/R injury with the potential to increase the number of liver suitable for liver transplantation.

2. Hepatic ischemia-reperfusion injury associated with liver transplantation. An unresolved problem in clinical practice

Liver transplantation (LT) dates back to 1963, when Thomas Starzl carried out the first transplant on a child suffering from biliary atresia. LT has evolved as the therapy of choice for patients with end-stage liver disease. However, I/R injury, inherent in every LT, is the main cause of both initial poor function and primary non-function of liver allograft. The latter is responsible for 81% of re-transplantations during the first week after surgery (Clavien et al., 1992; Jaeschke, 1996). I/R injury is a phenomenon whereby cellular damage in a hypoxic organ is accentuated following the restoration of oxygen delivery (Jaeschke, 1998; Teoh et al., 2003; Jaeschke, 2003). In the liver, this form of injury was recognized as a

clinically important pathological disorder by Toledo-Pereyra et al. in 1975 during studies of experimental LT. However, it was not until the mid-1980s that the term reperfusion injury was generally used in the literature on LT (Teoh et al., 2003).

A variety of clinical factors including starvation, graft age, and steatosis contribute to enhance liver susceptibility to I/R injury, further increasing the patient risks related to reperfusion injury (Shah & Kamath, 2003). In clinical LT, starvation of the donor, due to prolonged intensive care unit hospitalization or lack of an adequate nutritional support, increases the incidence of hepatocellular injury and primary nonfunction (Massip-Salcedo et al., 2007).

The waiting list for LT is growing at a fast pace, whereas the number of available organs is not growing at a proportional rate. The shortage of organs has led centers to expand their criteria for the acceptance of marginal grafts, which show poor tolerance to I/R (Busuttil & Tanaka, 2003). Some of these include the use of organs from aged donors, non-heart-beating donors (NHBD), and grafts such as small-for-size or steatotic livers. However, I/R injury is the underpinning of graft dysfunction that is seen in the marginal organ (Busuttil & Tanaka, 2003). The fundamental problem with NHBD organs is the prolonged warm ischemia before cold preservation (Reddy et al., 2004). Controlled NHBDs provide organs that are far less prone to ischemic damage and tend to offer superior posttransplant function (Busuttil & Tanaka, 2003). The use of uncontrolled NHBDs is associated with a very high risk of primary nonfunction (Reddy et al., 2004).

One of the benefits of reduced-size grafts from living donors is a graft of good quality with a short ischemic time, this latter being possible because live donor procurements can be electively timed with recipient procedure (Farmer et al., 2001). On the other hand, the major concern over application of living-related liver transplantation for adults is graft-size disparity. The small graft needs regeneration to restore the liver/body ratio. It is well known that I/R significantly reduces liver regeneration after hepatectomy (Franco et al., 2004).

Donor age of more than 70 years was found to be associated with lower patient and graft survival (Busuttil & Tanaka, 2003, Casillas et al., 2006). Additionally these donors also have an increased incidence of steatosis, which may potentiate cold preservation injury (Busuttil & Tanaka, 2003). Steatotic livers are one of the most common types of organs from marginal donors. The present review will focus on this type of liver grafts. Among other factors, unhealthy lifestyles associated with the consumption of alcohol and inappropriate diets have increased the proportion of patients with steatotic livers.

Hepatic steatosis is a major risk factor for liver surgery and transplantation, and fatty livers are unsuitable for many reasons. Operative mortality associated with steatosis exceeds 14%, compared with 2% for healthy livers, and the risks of primary non-function and dysfunction after surgery are similarly higher (Casillas et al., 2006; Selzner et al., 2000). Thus, hepatic steatosis is the major cause of graft rejection after LT and exacerbates the organ shortage problem (Fernández et al., 2004). Therefore, minimizing the adverse effects of I/R injury could increase the number of both grafts suitable for transplantation and patients who successfully recover from LT. The first step towards achieving this objective is a full understanding of the mechanisms involved in I/R injury.

3. Complexity of hepatic ischemia-reperfusion injury

A large number of factors and mediators play a part in liver I/R injury (Banga et al., 2005; Casillas et al., 2006; Fan et al., 1999; Jaeschke, 2003; Lentsch et al., 2000). The relationships between the signalling pathways involved are highly complex and it is not yet possible to describe, with absolute certainty, the events that occur between the beginning of reperfusion and the final outcome of either poor function or a non-functional liver graft.

Figure 1 shows some of the mechanisms involved in the pathophysiology of I/R injury. Due to the complexity of hepatic I/R injury, the present review summarizes the established basic concepts of the mechanisms and cell types involved in this process. The lack of oxygen to hepatocytes during ischemia causes mitochondrial deenergization, ATP depletion, alterations of H+, Na+, Ca2+ homeostasis that activate hydrolytic enzymes and impair cell volume regulation and sinusoidal endothelial cells (SEC) as well as Kupffer cells (KC) swelling (Massip-Salcedo et al., 2007). This fact together with the imbalance between nitric oxide (NO) and endothelin (ET) production, contributes to narrowing of the sinusoidal lumen and thus to microcirculatory dysfunction. Capillary narrowing also contributes to hepatic neutrophil accumulation (Peralta et al., 1996; Peralta et al., 2000a). Concomitantly, the activation of KC releases reactive oxygen species (ROS) and proinflammatory cytokines, including tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (Bilzer & Gerber, 2000; Lentsch et al., 2000). ROS can also derive from xanthine deshydrogenase/xanthine oxidase (XDH/XOD). Cytokines release throughout the induction of adhesion molecules (intercellular cell adhesion molecule [ICAM] and vascular cell adhesion molecule [VCAM]) and chemokines promote neutrophil activation and accumulation, thereby contributing to the progression of parenchymal injury by releasing ROS and proteases (Jaeschke, 1998, 2003; Lentsch et al., 2000). Besides, IL-1 and TNF-α recruit and activate CD4+ T-lymphocytes, which produce granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (INF-γ) and tumor necrosis factor beta (TNF-β). These cytokines amplify KC activation and TNF-α and IL-1 secretion and promote neutrophil recruitment and adherence into the liver sinusoids (Casillas et al., 2006; Selzner, 2003). Platelet activating factor (PAF) can prime neutrophils for superoxide generation, whereas leukotriene B4 (LTB4) contributes to the amplification of the neutrophil response (Jaeschke, 1998, 2003) (see Fig. 1).

The present review will present data from the literature about the possible sources of ROS, NO effects, mechanisms, and the role of some pro-inflammatory mediators such as TNF-α, and transcription factors, for example, nuclear factor kappa B (NFκB). These data will provide a better explanation on why hepatic I/R injury remains an unresolved problem in the clinical practice.

3.1 Mechanisms responsible for ROS production

The source of ROS in hepatic I/R has long been controversial. As regards the mechanisms responsible for ROS production, experiments with XDH/XOD inhibitors such as allopurinol suggest that this system is the main ROS generator in hepatocytes and it has also been implicated in LT-related lung damage (Casillas et al., 2006; Fernández et al., 2002). However, results obtained in experimental models of the isolated perfused liver have underestimated the importance of the XDH/XOD system, and suggest that mitochondria could be the main source of ROS (Jaeschke & Mitchell, 1989). On the other hand, some data challenge the

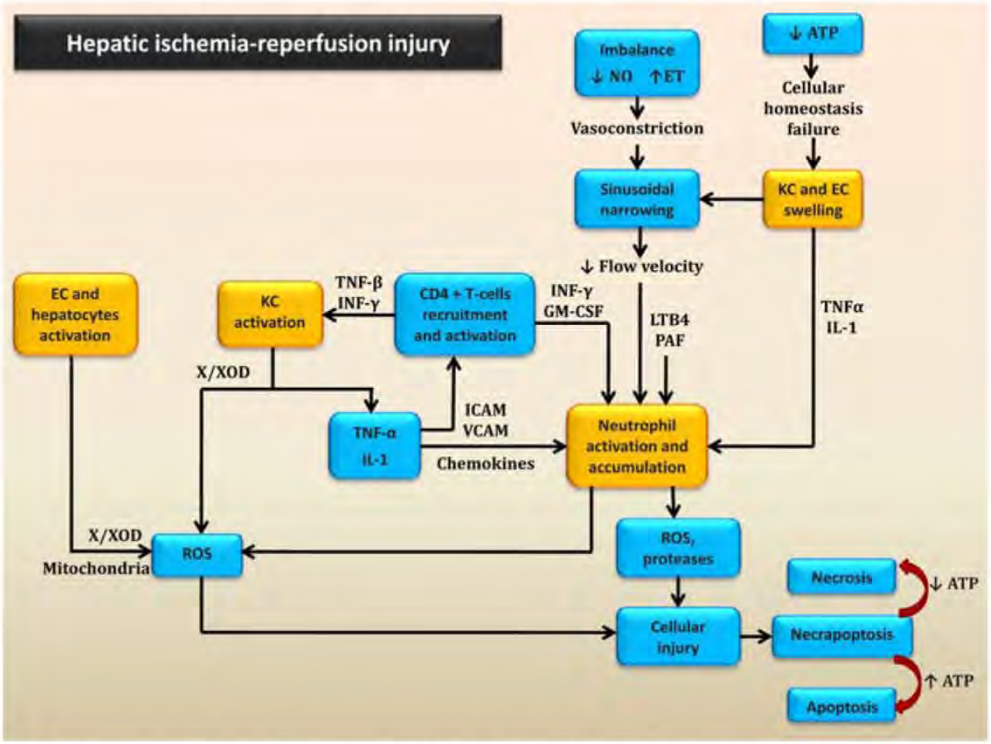

Fig. 1. Summary of the mechanisms involved in hepatic ischemia-reperfusion injury. (Bilzer & Gerber, 2000; Casillas et al., 2006; Jaeschke, 1998, 2003; Lentsch et al., 2000; Massip-Salcedo et al., 2007 ; Peralta et al., 1996, 2000a; Selzner, 2003)

pathophysiological relevance of intracellular oxidant stress during reperfusion (Grattagliano et al., 1999; Metzger et al., 1988). Grattagliano et al., 1999, demonstrated that mitochondria do not seem to actively participate in the reperfusion-induced oxidative stress. In addition, studies by Jaeschke et al. and Metzger et al. showed that the increased vascular oxidant stress after 30 and 60 min of ischemia was attenuated by inactivation of KC but not by high dose of allopurinol (Metzger et al., 1988). Interestingly, ROS release by KC occurs via the XDH/XOD system (Wiezorek et al., 1994). The conversion from XDH to XOD following cold storage is very slow in endothelial cells and hepatocytes, but much faster and higher in KC (Wiezorek et al., 1994). However, the KC function in I/R injury is still an area of active investigation. The elimination of KC did not modify the deleterious effects of I/R and the activation of neutrophils is not essential for reoxygenation injury (Imamura et al., 1995; Teoh et al., 2003). Clearly, then, there is a range of potentially conflicting results with regard to the mechanisms responsible for ROS generation in liver I/R injury. For instance, in our opinion, in order to clarify the importance of XDH/XOD versus mitochondria it should be taking into account that there are differences in the experimental models evaluated, including the times of ischemia. In this line, XDH/XOD play a crucial role in hepatic I/R injury only in conditions in which significant conversion of XDH to XOD occurs (80-90% of XOD) such as 16 h of cold ischemia. However, this ROS generation system does not appear to be crucial at

shorter ischemic periods such as 6 h of cold ischemia (Fernández et al., 2002). Thus, even after prolonged periods of ischemia, where a significant conversion of XDH to the XOD occurs, this enzyme may only play a minor role compared with mitochondria (Jaeschke & Mitchell, 1989). In contrast with the experimental studies, the clinical reports suggest that 45-65% XOD was sufficient to induce hepatic damage (Pesonen et al., 1998). In addition, the drugs used for inhibiting XDH/XOD should be considered, since, for example allopurinol, seems to have more than one mechanisms of action. It is not only a potent inhibitor of XOD, but it may also improve ischemia-induced mitochondrial dysfunction (Casillas et al., 2006; Jeon et al., 2001). In fact, evidence for reduced mitochondrial dysfunction after high doses of allopurinol was shown in a warm hepatic I/R model (Jeon et al., 2001). Similarly, in assessing the relative contribution of intracellular versus vascular oxidant stress to hepatic I/R injury, it should also be noted that oxidative stress in hepatocytes and the stimulatory state of KC after I/R depend on the duration of ischemia, and may also differ between ischemia at 4ºC and that at 37ºC, which probably leads to different developmental mechanisms of liver damage (Casillas et al., 2006). The differences in KC function in liver I/R injury cannot be attributed to the type experiment, since most authors used an *ex vivo* model of perfused rat liver. Nor could they be explained by differences in the times of cold ischemia, since the results obtained following the same ischemic period (24 h) were completely opposed (Imamura et al., 1995). The type of drug used for KC inactivation is the most probable explanation, since most of the studies implicating KC as main source of ROS used gadolinium chloride ($GdCl_3$) (Schauer et al., 2001; Zhong et al., 1996) whereas those that did not implicate KC used liposome-encapsulated dichloromethylene diphosphate (Imamura et al., 1995). Indeed, differences in the properties and action mechanisms of these two KC inhibitors have been reported.

3.2 Mediators and transcription factors in I/R injury

3.2.1 Nitric oxide

It is difficult to distinguish between beneficial and harmful mediators in I/R injury. Some authors have found that NO exerts a beneficial effect on I/R injury in different organs, tissues and cells, whereas other studies report no effect or even a deleterious action of NO (Peralta et al., 2001a). In our opinion, in addition to the differences in animal species, experimental models of hepatic I/R tested, and the dose and timing of administration of the different pharmacological modulators of NO, these differential effects of NO could be explained, at least partially, by the different source of NO. In this context, some studies suggest that although endothelial NO synthase (eNOS)-derived NO production is protective in I/R, inducible NO synthase (iNOS)-derived NO production may contribute to I/R injury. This may be a function of the NO generation kinetics of the two isoforms in I/R. The basal, low-level NO generation by the constitutively expressed eNOS isoform may abrogate the microcirculatory stresses of engraftment and reperfusion. In contrast, iNOS-derived NO cannot be generated until several hours after stimulation because of requirements for transcriptional induction of this isoform. Excess NO production may no longer be of microcirculatory benefit at this later time (Shah & Kamath, 2003). Furthermore, the excessive levels of iNOS-derived NO production may be detrimental through the generation of NOS-derived superoxide production or the generation of peroxynitrite. Additionally, whether NO is cytoprotective or cytotoxic in hepatic I/R injury may be determined at apoptosis (Casillas et al., 2006). For example, NO may promote apoptosis by inducing cytochrome c

(Cyt c) release and caspase activation (Chung et al., 2001). However, NO may also upregulate the anti-apoptotic protein Bcl-2 (Genaro et al., 1995). In addition, to understand the different results in relation with the action mechanisms of NO, it is important to clarify whether the NO source is endogenous or exogenous. In this regard, although the beneficial role of endogenous NO could be related to an attenuation of leukocyte accumulation, the exogenous supplementation of NO did not modify this parameter but was associated with an inhibition of endothelin release (Peralta et al., 2001a).

3.2.2 TNF and NFκB

Differential effects of NO mentioned above have also been reported for other mediators involved in hepatic I/R injury. According to the cell type and experimental or pathologic conditions, TNF-α is protective or injurious to the liver in the context of I/R injury. TNF-α may stimulate cell death or it may induce hepatoprotective effects mediated by antioxidant, antiapoptotic, and other anti-stress mediators coupled with a pro-proliferative biologic response (Casillas et al., 2006). For example, although the deleterious effect of the TNF-α in local and systemic damage associated with hepatic I/R is well established (Peralta et al., 1999), this mediator is also a key factor in hepatic regeneration (Teoh et al., 2003), an important process in reduced-size LT. Conversely, a study by our group found no correlation between TNF-α levels and liver regeneration in reduced-size LT (Franco et al., 2004), while Tian *et al.*, 2006, linked disruption of TNF-α release to lower hepatic injury and increased liver regeneration. These divergent results about the role of TNF-α in liver regeneration could be explained by different TNF-α inhibitors or animal species utilized in these experiments as well as differences in the experimental models of LT used, including the times of cold ischemia. These differential effects observed for TNF-α can also be extrapolated to transcription factors.

It is well known that NFκB can regulate various downstream pathways and thus has the potential to be both pro- and antiapoptotic (Fan et al., 1999). Currently it is not clear whether the beneficial effects of NFκB activation in protection against apoptosis or its detrimental proinflammatory role predominate in liver I/R (Fan et al., 1999). Hepatic neutrophil recruitment and hepatocellular injury are significantly reduced when NFκB activation is suppressed in mice following partial hepatic I/R (Casillas et al., 2006). However, nuclear factor kappa B (NFκB) activation is essential for hepatic regeneration after rat LT, and reduces apoptosis and hepatic I/R injury (Bradham et al., 1999). To understand the role of NFκB in the context of hepatic I/R, is important to consider the differences in animal species used, for instance, mechanisms of protection from apoptosis might be different in rats and mice (Chaisson et al., 2002). In addition, the experimental design used to evaluate the role of this transcription factor may also be important. Thus, some studies using adenoviral vector containing a repressor to prevent NFκB activation may not accurately reflect the role of NFκB signalling in regenerating liver because adenoviral vectors themselves cause increased TNF-α levels, DNA synthesis, and apoptosis in the liver before partial hepatectomy (Iimuro et al., 1998). Moreover, to explain these apparently controversial effects of NFkB, the pattern of NFkB activation under cold ischemia conditions should be taken into account. Takahashi *et al.*, 2002, have demonstrated in rat LT that NFkB activation during reperfusion occurs in two phases. The early peak of NFkB DNA binding was found 1-3 h after reperfusion and represents the nuclear translocation of NFkB p50/p65 heterodimers, whereas the second

peak, mainly composed of p50 homodimers, was observed at 12 h post-reperfusion. In this study, the donor liver treatment with adenovirus encoding the IkB super-repressor gene cannot affect the early peak of NFkB activation, but partially inhibited the second peak of NFkB DNA binding. The results indicated that, in contrast to early NFkB activation, inhibition of the late phase of NFkB activation was not associated with variations in levels of inflammatory mediators, but rather enhanced hepatocellular apoptosis (Takahashi et al., 2002), which reinforces the dual function of NFkB in transplanted liver. Nevertheless, this hypothesis does not fully explain the differences in the results. Indeed, Bradham *et al.*, 1999, observed a marked increase in apoptosis when NFkB blockade was carried out at 3 h of reperfusion, which seems to be a reperfusion time associated with the early peak of activation of NFkB. Of course, there are differences between Takahashi´s and Bradham´s studies. For example, whereas Bradham infused the adenoviral vector by endovenous injection 24 h before liver explantation, in Takahashi's study the graft was perfused with UW solution containing the adenovirus immediately before cold storage.

3.2.3 Neutrophil accumulation

Activation of neutrophils has been implicated in the hepatic microvascular dysfunction and parenchymal damage associated with I/R (Cutrin et al., 2002). Still, a controversial topic is the question of how neutrophils actually accumulate in the liver. The classical theory argues that the increased expression of adhesion molecules such as ICAM-1 and P-selectin plays a key role in neutrophil accumulation and the subsequent liver damage associated with I/R (Banga et al., 2005, Cutrin et al., 2002). In contrast, it has also been reported that neutrophil accumulation observed in the liver following I/R is not dependent on the up-regulation of either ICAM-1 or P-selectin (Peralta et al., 2001b).

To explain the results that neutrophil accumulation is not dependent on adhesion molecules, we subscribe to the theory proposed by Jaeschke, 2003. This theory argues that although P-selectin and ICAM-1 appear to be relevant for neutrophil adherence in postsinusoidal venules, the neutrophils relevant for the injury accumulate in sinusoids, which were identified as the dominant sites for neutrophil extravasation. In these capillaries, neutrophil sequestration does not depend on B2 integrins or on ICAM-1 or selectins (Essani et al., 1998; Vollmar et al., 1995; Jaeschke et al., 1996). Thus, mechanical factors such as active vasoconstriction, vascular lining cell swelling and injury, and reduced membrane flexibility after activation of the neutrophil, appear to be involved in trapping of these leukocytes in sinusoids (Jaeschke et al., 1996). The extensive vascular injury during reperfusion eliminates, in part, the sinusoidal endothelial cell barrier and the neutrophil has direct access to hepatocytes (Jaeschke, 2003; McKeown et al., 1988). Nevertheless, even with damaged but still present EC, transmigration may still be required (Jaeschke, 1998). As a consequence, I/R injury is only moderately or not at all attenuated by anti-ICAM therapies (Farhood et al., 1995; Vollmar et al., 1995). In regard with the role of P-selectin, sinusoidal EC neither contain Weibel Palade bodies nor do they transcriptionally upregulate relevant levels of P-selectin (Essani et al., 1998). However, during I/R, a number of interventions directed against selectins reduced hepatic neutrophil accumulation and cell injury (Amersi et al., 2001). Because these findings cannot be explained by the prevention of P-selectin-dependent rolling in sinusoids, it has been suggested that most liver I/R models include some degree of intestinal ischemia, which leads to neutrophil accumulation in remote organs including the liver (Casillas et al., 2006; Kubes et al., 2002). Thus the lower number of neutrophils in

the liver when selectins are blocked may be a secondary effect due to the protection of antiselectin therapy against intestinal reperfusion injury (Kubes et al., 2002).

3.3 Cell death in liver transplantation

The severity of hepatocyte damage depends on the length of time the ischemia lasts. In human LT, a long ischemic period is a predicting factor for post-transplantation graft dysfunction, and some transplantation groups hesitate to transplant liver grafts preserved for more than 10 h (Fernández et al., 2002). Some studies in experimental models of LT indicate that 24 h of cold ischemia induces low survival at 24 h after LT. However, at shorter ischemic periods, LT may also result in primary organ dysfunction. The main victims of ischemic injury are the hepatocytes and SECs. These two cell types show different responses to different types of ischemia: hepatocytes are more sensitive to warm ischemia and SECs to cold ischemia (Bilzer & Gerber, 2000; McKeown et al., 1988). Although most hepatocytes remain viable after 48 h of cold preservation and reperfusion, SECs suffer severe damage following reperfusion (40% non-viable) (Caldwell et al., 1989). The result of this sinusoidal damage is the subsequent microcirculatory abnormalities upon reperfusion, resulting in hepatocyte injury and dysfunction (McKeown et al., 1988). This contributes to the development of primary nonfunction or impaired primary function after LT. However, some studies have called the importance of sinusoidal injury into question. Huet et al., 2004, have demonstrated that damage to the extracellular matrix from prolonged preservation and reperfusion appears to be the critical factor in graft failure (Banga et al., 2005). In addition, it is possible that perturbations in hepatocyte levels of adenine nucleotides during cold storage can trigger proteolytic events that contribute to damage in the liver graft and subsequently compromise hepatic functions after LT (Kukan & Haddad, 2001). Moreover, cold ischemia profoundly disturb several key hepatocellular functions, such as volume and pH homeostasis, as well as solute transport and drug metabolism, protein synthesis and mitochondrial function. This contributes to preservation injury of the liver graft. Therefore, these observations indicate that aside from reducing EC damage, LT therapy may benefit from strategies aimed at improving the maintenance of appropriate hepatocyte functions (Kukan & Haddad, 2001; Vajdova et al., 2002).

Apoptosis has been regarded as the fate of cells experiencing I/R injury (Sasaki et al., 1996). In this line, different studies have demonstrated apoptotic death in hepatocytes and/or SECs after both cold and warm ischemia of the rat liver (Gao et al., 1998; Kohli et al., 1999). All of the aforementioned studies (Gao et al., 1998; Kohli et al., 1999; Sasaki et al., 1996) used TdT-mediated dUTP-biotin nick and labelling (TUNEL staining) for DNA ladders to demonstrate apoptosis. However, the ability of TUNEL staining to distinguish between apoptosis and necrosis has been called into question. The activation of caspases has also been used to demonstrate apoptosis in rat SECs following cold I/R (Natori et al., 1999). Indeed, use of pan-caspase inhibitors protected rat liver SECs and hepatocytes against I/R injury after prolonged periods of both cold and warm ischemia. On the other hand, other groups oppose the view that the majority of cells undergo apoptosis in response to either warm or cold I/R injury, believing that necrosis is the principle form of cell death (Massip-Salcedo et al., 2007). They believe that in a number of studies the proportion of cells undergoing apoptosis is not of significant magnitude and that the degree of caspase activation does not correlate with the number of SECs and hepatocytes supposedly undergoing apoptosis. Thus, a controversy has emerged over the past years as to whether

necrotic or apoptotic cell death accounts for the severe parenchymal injury observed during hepatic reperfusion. Although it has long been assumed that necrosis and apoptosis are different processes this may not actually be the case. First we will briefly review some basic background information on death cell signalling pathways in hepatocytes in order to understand the shared pathway that leads to both necrosis and apoptosis.

Apoptosis occurs through two main pathways. The first, referred to as the intrinsic (mitochondrial) pathway, is typically activated by a variety of stressors such as DNA damage, p53 activation, growth factor deprivation, and metabolic disturbances (Malhi et al., 2006). The second is the extrinsic pathway that is triggered through death receptors (Malhi et al., 2006). It is well known that one of the most important regulators of intrinsic pathway is the Bcl-2 family of proteins. The Bcl-2 family includes proapoptotic members such as Bax, Bak, Bad, Bid and antiapoptotic members such Bcl-2, Bcl-Xl and Bcl-W (Ghobrial et al., 2005). Following death signal, proapoptotic proteins undergo posttranslational modifications resulting in their activation and translocation to the mitochondria. Then, the outer mitochondrial membrane becomes permeable, leading to the release of Cyt c, which promotes caspase 9 activation, which then activates caspase 3 and the final stages of apoptosis (Ghobrial et al., 2005). In the extrinsic pathway, a variety of mediators, including tumor TNF-α, Fas ligand, and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) first bind to their respective death receptors, which cause receptor oligomerization and the association of various adapter proteins, including Fas-associated death domain, TNF-α receptor-associated death domain, and TNF-α receptor-associated factor. Fas-associated death domain and TNF-α receptor-associated death domain promote binding of procaspase 8 and its proteolytic activation to catalytic caspase 8. If sufficient amounts of caspase 8 are generated at the receptor, caspase 8 can directly activate procaspase 3. In hepatocytes the caspase 8 interacts with the intrinsic pathway and cleaves Bid, a BH3 only proapoptotic Bcl2 family member, to a truncated form, tBid. tBid translocates to mitochondria, causing mitochondrial permeabilization and release of mitochondrial effectors of apoptosis, such Cyt c (Yin, 2000) (see Fig. 2).

The mechanisms that induce the release of mitochondrial intermembrane proteins such as Cyt c remain controversial (Jaeschke & Lemasters, 2003). In hepatocytes TNF-α and Fas dependent signalling induce the onset of the mitochondrial permeability transition (MPT), which leads to large-amplitude mitochondrial swelling, rupture of the outer membrane, and release of Cyt c and other proteins from the intermembrane mitochondrial space (Jaeschke & Lemasters, 2003). In some models, tBid interaction with either Bax or Bak, forms channels in the mitochondrial outer membrane that release Cyt c and other proteins from the intermembrane space. If MPT onset occurs in relatively few mitochondria, the organelles become sequestered into autophagosomes for lysosomal digestion, a process that eliminates the damaged and potentially toxic mitochondria (Casillas et al., 2006; Jaeschke & Lemasters, 2003). When the MPT involves more mitochondria, mitochondrial swelling leads to outer membrane rupture and Cyt c realease. Provided that ATP is available from glycolysis and still-intact mitochondria, Cyt c activate downstream caspases and other executioner enzymes of apoptosis. When MPT onset is abrupt and involves most mitochondria, ATP becomes profoundly depleted, which blocks caspase activation. Instead, ATP depletion culminates with plasma membrane rupture and the onset of necrotic cell death (Jaeschke & Lemasters, 2003). Hence, the new term "necrapoptosis" has been coined to describe a process that begins with a common death signal and which culminates in either cell lysis

(necrotic cell death) or programmed cellular resorption (apoptosis), depending on factors such as the decline of cellular ATP levels (see Fig. 2).

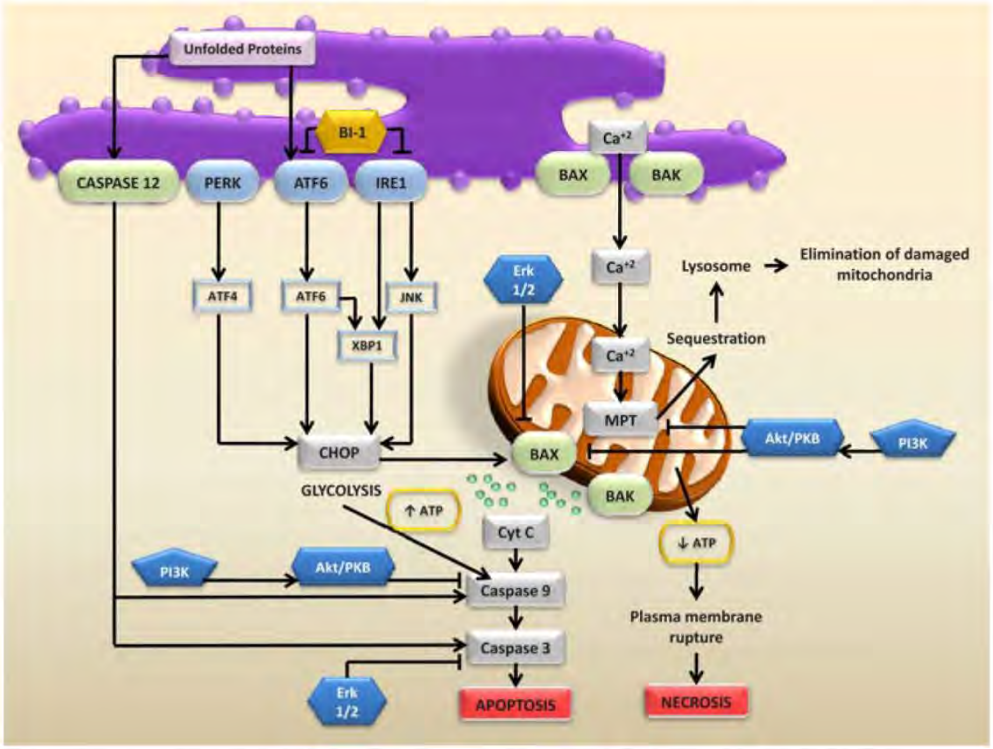

Fig. 2. Scheme of possible cell death pathway in hepatic I/R. (Alfany et al., 2009; Ben Mosbah et al., 2010; Casillas et al., 2006; Fernández et al., 2004; Ghobrial et al., 2005; Jaeschke &Lemasters, 2003; Malhi et al., 2006; Massip-Salcedo et al., 2007; Selzner et al., 2000; Yin, 2000)

4. Steatosis in hepatic ischemia-reperfusion

Several hypotheses have been suggested to explain the decreased tolerance of steatotic liver to I/R injury compared with non-steatotic livers. The impairment of the microcirculation is considered a major event of reperfusion injury in steatotic livers (Ijaz et al., 2003). A reduction in hepatic microcirculation has been observed in human fatty donor livers and in experimental models of hepatic steatosis (Ijaz et al., 2003; Seifalian et al., 1999). An imbalance between vasoconstrictors (e.g., ET1) and vasodilators (e.g., NO) negatively affect the hepatic microcirculation (Massip-Salcedo et al., 2007; Peralta et al., 2000a). In addition, fatty accumulation in the cytoplasm of hepatocytes is associated with an increase in cell volume that reduces the size of the hepatic sinusoid space by 50% compared with a normal liver and may result in partial or complete obstruction of the hepatic sinusoid space (Ijaz et al., 2003; Seifalian et al., 1999). Using Doppler flowmetry, Seifalian et al., 1999 demonstrated reduced sinusoidal perfusion in fatty human liver donors compared with healthy livers.

Analogous studies in rabbits with diet-induced steatosis confirmed that this reduction in perfusion correlated with the severity of fat accumulation in hepatocytes. The reductions in sinusoidal perfusion appear to arise initially from the effects of enlarged hepatic parenchymal cells, swollen with accumulated lipid, which widen the parenchymal cell plates and narrow and distort the lumens of sinusoids. Other investigators have shown that as a result of the structural alterations around them, the sinusoids become inefficient conduits of blood with resulting impairment of tissue perfusion, evidenced by the significant reductions in the numbers of perfused sinusoids per microscopic field (Teoh et al., 2010).

Hepatocyte damage appears remarkably higher in steatotic livers than in non-steatotic livers (Casillas et al., 2006; Selzner et al., 2000). Several evidences indicate that an increased sensitivity of fatty hepatocytes to the injurious effects of ROS could explain the poor tolerance of steatotic livers to I/R (Koneru et al., 2005; Soltys et al., 2001). It has been postulated that steatotic livers are more susceptible than nonsteatotic livers to lipid peroxidation because of either their lower antioxidant defenses or their greater production of ROS or both (Fernández et al., 2004). Mitochondrial ROS generation dramatically increases during reperfusion and mitochondrial structures are exposed to the attack of the ROS generated both outside and inside these organelles leading eventually to the dysfunction of important mitochondrial processes including those responsible for the ATP synthesis. In ROS generation systems, the inhibition of XOD with allopurinol effectively protected against the greater liver and lung damage in transplantation of steatotic livers (Fernández et al., 2004). Higher levels of IL-1β and lower IL-10 levels were observed in steatotic livers compared with non-steatotic livers after I/R. This imbalance between pro- and anti-inflammatory ILs was responsible for the vulnerability of steatotic livers to I/R (Serafin et al., 2004). Previous studies form our group indicated less glutathione (GSH) and SOD levels in steatotic livers than in non-steatotic livers as consequence of hepatic I/R (Fernández et al., 2004; Serafin et al., 2002).

It is well-known that steatotic livers synthesise less ATP than non-steatotic livers during post-ischemic reperfusion (Caraceni et al., 2005). Fatty degeneration induces a series of ultra-structural and biochemical alterations in both human and animal mitochondria. The lower ATP and adenine nucleotide content observed in steatotic livers preserved in UW solution could be caused by mitochondrial damage (Ben Mosbah et al., 2006; Caraceni et al., 2005; Massip-Salcedo et al., 2007). Caraceni et al., 2004 reported that alterations in oxidative phosphorylation during preservation is greatly enhanced by fatty infiltration resulting from damage to respiratory chain complex I and F0F1–ATP synthase. Others studies have discovered that in steatotic livers under conditions of either warm ischemia or transplantation, the content of mitochondrial uncoupling protein-2 (UCP-2) is four to five times higher than in non-steatotic livers (Chavin et al., 2004; Wan et al., 2008). This finding was associated with reduced ability to synthesize ATP upon reperfusion (Chavin et al., 2004). If cold storage time exceeds 10-12 h, complications in biliary structures occur in more than 25% of liver transplant recipients (Kukan & Haddad, 2001). Several factors, including poor recovery after ATP depletion appear to contribute to bile duct cell damage after liver transplantation. Furthermore, isolated rat bile duct epithelial cells are noticeably sensitive to oxidative stress, possibly because their cellular stores of reduced glutathione are seven times lower than those of hepatocytes (Noack et al., 1993). Taking these observations into account, bile production failure in steatotic livers could be explained, at least partially, by the lower

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It is well-known that steatotic livers synthesise less ATP than non-steatotic livers during post-ischemic reperfusion (Caraceni et al., 2005). Fatty degeneration induces a series of ultra-structural and biochemical alterations in both human and animal mitochondria. The lower ATP and adenine nucleotide content observed in steatotic livers preserved in UW solution could be caused by mitochondrial damage (Ben Mosbah et al., 2006; Caraceni et al., 2005; Massip-Salcedo et al., 2007). Caraceni et al., 2004 reported that alterations in oxidative phosphorylation during preservation is greatly enhanced by fatty infiltration resulting from damage to respiratory chain complex I and F0F1–ATP synthase. Others studies have discovered that in steatotic livers under conditions of either warm ischemia or transplantation, the content of mitochondrial uncoupling protein-2 (UCP-2) is four to five times higher than in non-steatotic livers (Chavin et al., 2004; Wan et al., 2008). This finding was associated with reduced ability to synthesize ATP upon reperfusion (Chavin et al., 2004). If cold storage time exceeds 10-12 h, complications in biliary structures occur in more than 25% of liver transplant recipients (Kukan & Haddad, 2001). Several factors, including poor recovery after ATP depletion appear to contribute to bile duct cell damage after liver transplantation. Furthermore, isolated rat bile duct epithelial cells are noticeably sensitive to oxidative stress, possibly because their cellular stores of reduced glutathione are seven times lower than those of hepatocytes (Noack et al., 1993). Taking these observations into account, bile production failure in steatotic livers could be explained, at least partially, by the lower

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ATP and increased oxidative stress presented by this type of liver compared with non-steatotic liver.

Toll-like receptor 4 (TLR4) has been implicated as a mediator of steatotic liver damage after I/R (Ellett et al., 2009). The loss of TLR4 in steatotic livers from TLR4-knockout HFD animals reduces pro-inflammatory cytokines and liver injury and improves survival (Ellett et al., 2009). Although TLR4 signaling is relevant in hepatic I/R injury, there is some controversy over which of the pathways [(myeloid differentiation factor 88 (My-D88)-dependent) or Toll/IL-1 receptor domain-containing adaptor inducing interferon-β (TRIF/IRF-3 signalling pathway)] is activated in hepatic I/R (Kang et al., 2011). Neutrophils have been involved in the increased vulnerability of steatotic livers to I/R injury, especially in alcoholic steatotic livers. However, neutrophils do not account for the differentially greater injury in the non-alcoholic steatotic liver during the early or late hours of reperfusion. Similarly, the role of TNF-α in the vulnerability of steatotic livers to I/R injury may be dependent on the type of steatosis (Serafin et al., 2002). These observations could be of clinical interest because pharmacological strategies that could be effective in alcoholic fatty livers by reducing the neutrophil infiltration and or TNF-α action may not be sufficient to reduce the hepatic I/R injury in non-alcoholic fatty livers.

Cell death can occur by either necrosis or apoptosis and intracellular ATP level appear to play a role as a putative apoptosis/necrosis switch: when ATP depletion is severe, necrosis ensues before the activation of the energy-requiring apoptotic pathway (Casillas et al., 2006; Massip-Salcedo et al., 2007) (See Fig. 2). In steatotic liver graft undergoing 6 h of cold ischemia, necrosis was the predominant cell death whereas no apoptosis signs were found (Alfany et al., 2009; Fernández et al., 2004). Since apoptosis is an energy-requiring process, the impaired maintenance of ATP levels observed after reperfusion in steatotic livers submitted to long periods of cold ischemia may be linked with a failure to induce apoptosis. Thus, it is not surprising that data reported previously indicate that necrosis rather than apoptosis is the predominant process by which steatotic livers undergo cell death (Alfany et al., 2009; Fernández et al., 2004; Selzner et al., 2000).

Previous studies from our group have indicated that steatotic livers differed from non-steatotic livers in their response to UPR and ER stress. Steatotic livers showed a reduced ability to respond to ER stress as the activation of two UPR arms, IRE1 and PERK, was weaker in the presence of steatosis. (Ben Mosbah et al., 2010). Different hypotheses, including decreased ATP production and dysfunction of regulators of apoptosis, such as Bcl-2, Bcl-xL and Bax have been proposed to explain the failure of apoptosis in steatotic livers. The results on ER stress in steatotic livers undergoing I/R may throw some light on this question. Reduced proapoptotic factors related to ER stress such as caspase 12, C/EDP-homologos protein (CHOP) and Jun N-terminal kinase (JNK) were observed in steatotic livers under conditions of I/R compared with non-steatotic livers. This may be related to the reduced activation of the two UPR arms, inositol-requiring enzyme-1 (IRE1) and PERK, which are responsible for caspase 9 and 12 activation, JNK activation and CHOP induction (Ben Mosbah et al., 2010) (see Fig. 2). We believe that the damaged ER and mitochondria are intimately linked and that mitochondrial cell death and ER-induced cell death cannot be separated in hepatic I/R. Thus, caspase activation and Cyt *c* release from mitochondria consequently to hepatic I/R (Ben Mosbah et al., 2010) can be attributed to ischemic disturbance or damage to the ER. Given these results in steatotic livers under warm

ischemia conditions, it is therefore tempting to speculate that increased ER stress may be involved in the vulnerability of steatotic liver grafts to I/R injury associated with transplantation and in the sensitivity of other marginal grafts to I/R injury, such as liver grafts from aging donors. Indeed, aging donors have an increased incidence of steatosis, which may favor cold preservation injury (Busuttil & Tanaka, 2003; Massip-Salcedo et al., 2007). Alterations in the activation of inflammatory transcription factors and expression of cytoprotective proteins, increased intracellular oxidants and decreased mitochondrial function and protein misfolding accumulation, and aggregation also characterize many age-related diseases (Massip-Salcedo et al., 2007; Pallet et al., 2009).

5. Strategies designed in past years to prevent hepatic I/R injury

Despite improvements in pharmacological treatments, preservation solutions and gene therapy aimed at reducing hepatic I/R injury, the results to date have not been conclusive. Figure 3 shows some of the therapeutic strategies developed to prevent I/R injury in LT. Possible reasons for the failure of these strategies in clinical applications are now discussed.

5.1 Pharmacological treatment

Numerous experimental studies have focused on inhibiting the harmful effects of I/R-associated inflammatory response. In this respect, drugs such as chloroquine and chlorpromazine have been administered in order to prevent mitochondrial dysfunction and loss of liver cell phospholipids during hepatic ischemia. Antioxidant therapy using either tocopherol, GSH ester, or allopurinol has been applied in an attempt to inhibit ROS effects in reperfusion, and anti-TNF antiserum pre-treatment has also been employed to block the damaging effects of this cytokine. Therapies with dopamine or ATP-$MgCl_2$ have been administered to reduce hepatic I/R injury-related microcirculatory disorders. Drugs such as adenosine, NO donors, L-arginine, and anti-ICAM-1 and anti-P-selectin antibodies have been used to inhibit neutrophil accumulation. However, none of these treatments has managed to prevent hepatic I/R injury. The possible side effects of the some drugs may frequently limit their use in human LT (Casillas et al., 2006). For example, idiosyncratic liver injury in humans is documented for chlorpromazine, pernicious systemic effects have been described for nitric oxide (NO) donors, allopurinol therapy can cause haematological changes and gadolinium can induce coagulation disorders (Casillas et al., 2006).

Hepatic failures have been observed after administration of these two thiazolidinediones (TZDs) and some case reports of acute hepatotoxicity attributed to rosiglitazone have been published, including one death (Reynaert et al., 2005). The toxicity of TZDs is thought to be mainly metabolic idiosyncratic, although in some cases possible immunological mechanism has been implicated (Reynaert et al., 2005). High dose resveratrol was found to be a pro-oxidant with aggravation of liver injury; and experiments are in progress to devise a pharmaceutical form appropriate for clinical use (Hassan et al., 2008). The development of therapeutic strategies that utilize the protective effect of Heme oxigenase-1 (HO-1) induction is hampered by the fact that most pharmacological inducers of this enzyme perturb organ function by themselves and that gene therapy for up-regulation of HO-1 has potential negative side effects, which currently preclude its clinical application under these conditions (Schmidt, 2010) (see Fig. 3).

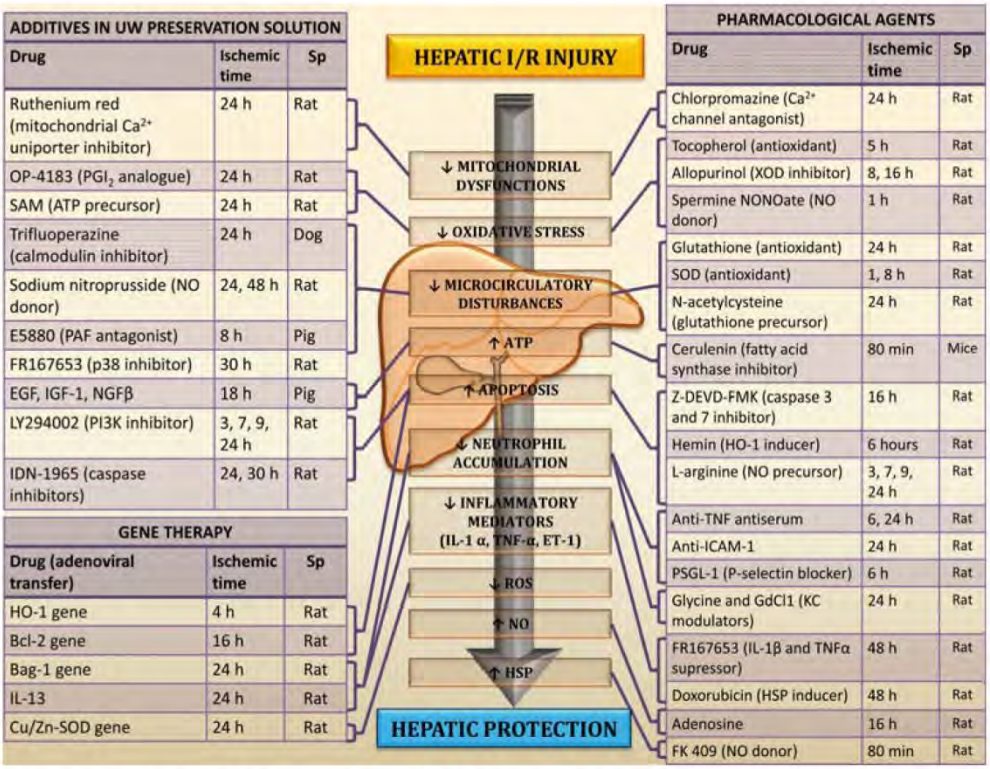

Fig. 3. Strategies used to prevent hepatic I/R injury. (Sp, species). (Carini et al., 2004; Carrasco et al., 2005; Casillas et al., 2006; Chavin et al., 2004; Cheng et al., 2003; Esfandiari et al., 2007; Fan et al., 1999; Hassan et al., 2008; Massip-Salcedo et al., 2006; Nakano et al., 2007; Natori et al., 1999; Peralta et al., 2001a, 2001b; Polyak et al., 2000; Reynaert et al., 2005; Schmidt, 2010; Selzner et al., 2000, 2003; Teoh et al., 2003; Vajdova et al., 2002; Yoshinari et al., 2001)

The difficulty of blocking the inflammation related to this process must be taken into account because, among other factors, many mediators and cell types are involved in this kind of inflammatory response. Pharmacological treatment-derived difficulties must also be considered. In this regard, superoxide dismutase (SOD) and glutathione show inadequate delivery to intracellular sites of ROS action (Polyak et al., 2000). The administration of anti-TNF antibodies does not effectively protect against hepatic I/R injury, and this finding has been related to the failure of complete TNF-α neutralization locally (Peralta et al., 2001b). Additionally, special attention should be given to drugs that suppress TNF-α, because its potential dual effects (Teoh et al., 2003). Small changes in the dose of NO donors produce totally opposite effects (Peralta et al., 2001a). Although this also occurs in non-steatotic livers, modulating I/R injury in steatotic livers poses a greater problem. Until now, data about the effectiveness of the administration of antioxidants on the deleterious effects of ROS in steatotic livers was controversial. Some studies in obese Zucker rats, a well-characterized model of nutritionally induced obesity, indicated that the administration of

tocopherol, which possesses antioxidant properties, improved tolerance to warm ischemia. However, other experimental studies in steatotic livers, induced by a choline–methionine-deficient diet, show that the administration of GSH precursors, such as N-acetylcysteine, could help to restore hepatocellular integrity in the steatotic liver but without scavenging free radical. In addition, both dietary high fat and alcohol exposure produced SOD/catalase-insensitive ROS that may be involved in the mechanism of failure of steatotic livers after orthotopic LT (Casillas et al., 2006; Massip-Salcedo et al., 2007; Serafin et al., 2002; Soltys et al., 2001).

Differences in the action mechanisms between steatotic and non-steatotic livers mean that therapies which are effective in non-steatotic livers may prove useless in the presence of steatosis, and the effective drug dose may differ between the two liver types. Findings such as these must be taken into consideration when applying pharmacological strategies in the same way to steatotic and non-steatotic livers, because the effects may be very different. Apoptosis was the predominant form of hepatocyte death in the ischemic non-steatotic liver, whereas the steatotic livers developed massive necrosis after an ischemic insult. Thus, caspase inhibition, a highly protective strategy in non-steatotic livers, had no effect on hepatocyte injury in steatotic livers (Selzner et al., 2000). For instance, whereas in an LT experimental model a NO donor reduced oxidative stress in non-steatotic livers, the same dose increased vulnerability of steatotic grafts to I/R injury (Carrasco et al., 2005). The injurious effects of exogenous NO donors on hepatic injury and oxidative stress in steatotic grafts could be explained by peroxinitrite generation caused by ROS overproduction (Carrasco et al., 2005). HO-1 activators such as cobalt (III) protoporphyrin IX, might protect both liver types against warm I/R injury. However, a lower dose of HO-1 activator was required to protect steatotic livers effectively, as steatotic livers undergoing I/R showed higher HO-1 levels than nonsteatotic livers (Massip-Salcedo et al., 2006). Furthermore, there may be drugs that would only be effective in steatotic livers. In the context of LT, steatotic donors have been reported to show a higher content of mitochondrial uncoupling protein-2 (UCP-2) and a reduced ability to synthesize ATP upon reperfusion, thus leading to increased mortality following I/R (Cheng et al., 2003). Studies by Chavin *et al* have discovered that in ob/ob mice (approximately 70%-80% of liver lipid content) expression of UCP-2 is four to five times higher than in normal liver tissues (Chavin et al., 1999; Wan et al., 2008). Hence, compounds such as cerulenin that reduce UCP-2 expression in steatotic livers, offer protection as a result of increased availability of ATP prior to I /R (Chavin et al., 2004). However, this strategy may be ineffective in non-steatotic livers because the latter do not show an overexpression of UCP-2 (Chavin et al., 1999). Similar results have been obtained with carnitine administration (Tolba et al., 2003; Yonezawa et al., 2005).

All the aforementioned results point up the fact that the different mechanisms of cell death in steatotic vs. non-steatotic livers as well the differences in the mechanisms involved in hepatic I/R injury in terms of the type of steatosis could explain the difficulties in effectively preventing steatotic livers from I/R injury. Further investigations are required to optimize some treatments because long-term therapy appears to be necessary to exert the desired effects. For example, the pre-treatment times for rosiglitazone was between 6 to 12 weeks (Nakano et al., 2007); and, S-adenosylmethionine (SAM) between 14 and 17 weeks (Esfandiari et al., 2007). Similarly, long-term IL-6 treatment (10 days) reduced hepatic steatosis and markedly prevents I/R-induced liver injury in ob/ob mice and mice fed high-

fat diets (Hong et al., 2004). However, there are obvious difficulties concerning the feasibility of long-term drug administration in some I/R processes, in particular, liver transplantation from cadaveric donors, because this is an emergency procedure in which there is very little time to pre-treat the donor with drugs.

5.2 Preservation solutions

Since its introduction by Belzer et al. in the late eighties, the University of Wisconsin (UW) solution has become the standard solution for the preservation of most organs in transplantation. The inclusion of some components in the UW solution has been both advocated and criticised. For instance, adenosine has been added to UW solution as a substrate for the regeneration of adenine nucleotides. However, simplified variants of UW solution in which adenosine was omitted were shown to have similar or even higher protective potential during cold liver storage. The colloid hydroxyethyl starch (HES) included in UW preservation solution prevents interstitial edema but produces extended and accelerated aggregation of erythrocytes that may result in stasis of blood and incomplete washout of donor organs before transplantation. Another limitation of the UW solution is that some of its constituent compounds (allopurinol, lactobionate) do not offer very good protection because they are not present at a suitable concentration and encounter problems in reaching their site of action. Indeed, studies in humans have suggested that the allopurinol in the UW preservation solution was unable to prevent the subsequent XDH/XOD-derived superoxide radical production during reperfusion (Casillas et al., 2006; Pesonen et al., 1998).

A variety of ingredients such as stable protacyclin (PGI2) analogue OP-4183, p38 mitogen-activated protein kinase (MAPK) inhibitor FR167653, NO donor sodium nitroprusside, platelet-activating factor (PAF) antagonist E5880, calmodulin inhibitors, Ca^{2+} channel blockers such as nisoldipine, trophic factors, caspase or calpain inhibitors, S-adenosylmethionine (SAM), insuline, or fructose-1,6-biphosphate (FBP) were introduced into UW preservation solution, with promising results (Casillas et al., 2006). However, none of these modifications to UW solution composition have found their way into routine clinical practice. For instance, studies aimed at enrichment of UW solution with caspase inhibitors showed that this prevents sinusoidal endothelial cells apoptosis (Vajdova et al., 2002), but it has also been demonstrated that such inhibitors have little effect on necrosis, and this could mean no protection in the steatotic liver where the predominant form of cell death is necrosis (Selzner, 2003). Along this line, addition of precursors for ATP resynthesis such as SAM only resulted in a poor initial ATP recovery during liver reperfusion (Vajdova et al., 2002) (see Fig. 3). Insulin and FBP were recommended and added to UW preservation solution with the aim of stimulating glycolysis and modulating KC activity, respectively. However, further studies showed that these modifications in UW solution may exacerbate graft ischemic injury and decrease the graft survival rate in rat LT.

The failure of UW solution enrichments could be related either to factors intrinsic to the drugs themselves (i.e. toxic side-effects, lack of specificity, etc.) or disagreement in their mechanisms of modulation. For instance, LY294002 was added to UW in order to maintain calcium homeostasis through the inhibition of phosphatidylinositol-3-OH kinase (PI3K) activity (see Fig. 3). Despite LY294002 reduces apoptosis in the grafts, the beneficial effects of the survival pathway activated by PI3K were also suppressed (Carini et al., 2004).

Additives to UW solution might further improve survival rate and graft viability if their concentration could be increased, but this is not always possible. For example, the solubility of FR167653 in UW solution was found to be limited. In addition, these additives are rinsed from the liver graft before implantation, so they should have prolonged action (Yoshinari et al., 2001). For instance, addition of precursors for ATP re-synthesis, such as S-adenosylmethionine, only resulted in a poor ATP recovery during reperfusion, since they can be rescued only partially after liver flush before implantation (Vajdova et al., 2002). Another limitation is that suitable concentrations of additives, such as caspase inhibitor IDN-1965, can be achieved only with prolonged storage of the organ in the presence of the inhibitor (Natori et al., 1999). However, this exacerbates the cold ischemic injury.

Numerous studies have reported equivalent patient and graft survival for deceased donor liver allografts preserved with UW and HTK solutions (Steawart et al., 2009). The reduced viscosity of HTK as compared to UW has been hypothesized to be protective against the development of biliary complications. However, the impact of HTK versus UW preservation on biliary complications remains unclear, as some centers report equivalent, increased or reduced rates of biliary complication with HTK preservation of deceased donor liver allografts (Feng et al., 2007; Steawart et al., 2009).

Clinical studies indicated that HTK preservation was associated with higher odds or early graft loss as compared to UW preservation with a more pronounced effect on allograft with cold ischemia time over 8 h, donor after cardiac death allografts and donors over 70 years (Steawart et al., 2009). As previously reported, HTK is not so efficient for longer periods of cold ischemia causing a higher incidence of delayed graft function (Olschewski et al., 2008; Straatsburg et al., 2002)

5.3 Gene therapy

Advances in molecular biology provide new opportunities to reduce liver I/R injury by using gene therapy. To suppress the ROS burst, SOD and catalase have been transfected by either adenovirus, liposomes or polyethyleneglycol (Fan et al., 1999; Selzner, 2003). To inhibit apoptosis, overexpression of Bag-1 and Bcl-2, mainly by using adenovirus, has been tested (Selzner, 2003) (see Fig. 3). To limit neutrophil recruitment and activation, reduction in ICAM-1 expression was obtained by using liposomes. Cytoprotective strategies based on expression of genes such as HO-1, anti-inflammatory cytokine IL-13 and interleukin-1 receptor antagonist (IL-1Ra) have been developed employing adenoviral or liposome vector (Casillas et al., 2006). Attempts have also been made to modulate the NFκB effect through adenoviral transfection of a mutant inhibitor of kappaB-alpha (IκBalpha), which would inhibit NFκB and ameliorate the hepatic inflammatory response to I/R (Fan et al., 1999; Casillas et al., 2006) (see Fig. 3). However, there are a number of problems inherent in gene therapy, for example, vector toxicity, difficulties in increasing transfection efficiencies and protein expression at the appropriate time and site, and the problem of obtaining adequate mutants (in the case of NFκB) due to controversy about NFκB activation (Chaisson et al., 2002; Somia & Verma, 2000). Although non-viral vectors (such as naked DNA and liposomes) are likely to present fewer toxic or immunological problems, they suffer from inefficient gene transfer (Somia & Verma, 2000). In addition, LT is an emergency procedure in most cases, which leaves very little time to pre-treat the donor with genetic approaches.

6. Directions for the future

New potential strategies that could be promissory in LT are now discussed. The present review will now centre on emerging protective strategies such as enrichments of UW solution and pharmacological treatments with favourable results in I/R injury but that up to now have not been tested in clinical LT. Moreover, we will discuss ischemic preconditioning taking into account the novel clinical reports that suggest the effectiveness of this surgical procedure in LT.

6.1 Pharmacological treatments and preservation solutions

6.1.1 Trimetazidine and AICAR

Trimetazidine (TMZ), which has been used as an anti-ischemic drug in the heart for over 35 years (Ikizler et al., 2003) reduced liver injury and improved liver regeneration and survival rate in partial hepatectomy under vascular occlusion (Casillas et al., 2006). TMZ has been used as an additive in UW solution to protect steatotic livers exposed to prolonged cold ischemia in an *ex vivo* model of hepatic ischemia (Ben Mosbah et al., 2006). This could be of interest since irreversible injury has been reported in liver grafts preserved in UW after prolonged cold ischemic periods (between 16 h to 24 h) (Ben Mosbah et al., 2006). Studies examining the underlying protective mechanisms of TMZ suggest that mitochondria, energy metabolism, oxidative stress and microcirculation might be important targets through which TMZ exerts its cytoprotective effect (Ben Mosbah et al., 2006; Ikizler et al., 2003). Interestingly, these mechanisms are responsible for the vulnerability of steatotic livers to I/R. Similarly to the benefits of TMZ, the addition of AMPK activators to UW solutions such as 5-amino-4-imidazole carboxamide riboside (AICAR), protected steatotic livers against their vulnerability to I/R. TMZ, by means of AMPK, increased NO, thus protecting steatotic livers against their vulnerability to I/R injury (Ben Mosbah et al., 2006, 2007; Carrasco et al., 2005). Taking these observations into account, TMZ and AICAR may constitute new additives to UW solution in steatotic liver preservation, whereas a combination of both seems unnecessary.

6.1.2 Modulators of renin-angiotensin system

Previous researches have observed an important role for the renin-angiotensin system (RAS), known for its regulation of blood pressure and fluid homeostasis, in both I/R injury and liver regeneration after partial hepatectomy (Ramalho et al., 2002; 2009). Furthermore, angiotensin-converting enzyme (ACE) inhibitors (captopril and enalapril) and angiotensin II (Ang-II) type 1 receptor blockers (losartan and candesartan) reduced inflammatory response associated with I/R injury (Araya et al., 2002). In addition, ACE inhibitors (lisinopril, captopril and enalaprilat) promoted liver regeneration after partial hepatectomy (Ramalho et al., 2002). Candesartan, a potent and long-lasting Ang-II type 1 receptor antagonist, up-regulated the hepatocyte growth factor (HGF), the most potent mitogen for mature hepatocytes (Araya et al., 2002). Steatotic livers against I/R. In conditions of partial hepatectomy under I/R, Angiotensin receptors (AT1R and AT2R) antagonists for steatotic livers improved regeneration in the remnant liver. AT1R antagonist, through NO inhibition, protected steatotic livers against oxidative stress and damage. The combination of AT1R and AT2R antagonists in steatotic livers showed stronger liver regeneration than either

antagonist used separately and also provided the same protection against damage as that afforded by AT1R antagonist alone. These results could be of clinical interest in liver surgery (Ramalho et al., 2009). BK seems to be a key mediator in the benefits of all the blockers of Ang II activity (ACE inhibitors, AT1R antagonists, and AT2R antagonists) in steatotic livers undergoing I/R (Casillas et al., 2008). In liver transplantation, Ang II is an appropriate therapeutic target only in non-steatotic livers. It was observed an upregulation of ACE2 in steatotic liver grafts, which was associated with decreased Ang II and high Ang-(1–7) levels. Ang-(1–7) receptor antagonist reduced necrotic cell death and increased survival in recipients transplanted with steatotic liver grafts. These results indicate a novel target for therapeutic interventions in liver transplantation within the RAS cascade, based on Ang- (1–7), which could be specific for this type of liver (Alfany et al., 2009). Further studies will be required to elucidate whether these strategies based on regulating RAS can be useful in hepatic I/R injury. ACE inhibitors are widely used in clinical practice. However, hepatotoxicity and cholestatic liver diseases have been reported under ACE inhibition (Casillas et al., 2008). Previous studies have indicated that losartan is as effective as captopril in its cardiovascular effects but has fewer adverse effects (Zhu et al., 2000). Thus, AT1R antagonists may be a safer protective pharmacologic strategy than ACE inhibitors for hepatic I/R injury.

6.1.3 Modulators of activating pro-survival kinase cascades, PI3K-Akt and Erk 1/2 pathway

Trophic factors such as insulin-like growth factor (IGF), EGF, cardiotrophin-1 and fibroblast growth factor (FGF) have been shown to protect against I/R injury through the activation of phosphatidylinositol-3-OH kinase (PI3K)-Akt and p42/p44 extra-cellular signal-regulated kinases (Erk 1/2). This pathway has been implicated in cellular survival, through recruitment of anti-apoptotic protection pathways. PI3K-Akt has been shown to increase NO, inhibit opening of the MPT pore, and activate protein kinase C (PKC) and mitochondrial Raf-1, which has been shown to phosphorylate and inactivate the pro-apoptotic factor, Bad. Activation of either the PI3K-Akt or the Erk 1/2 pathway inhibits the conformational change in Bax required for its translocation to the mitochondria. Moreover Erk 1/2 kinase activation has been shown to inhibit apoptosis, by inhibiting caspase 3 activation and Akt activation can suppress the mitochondrial apoptotic death pathway by inactivating caspase 9. Interestingly, PI3K-Akt is a cell signalling mechanism also involved in the benefits of liver ischemic preconditioning in isolated hepatocytes. The modulation of therapeutic targets such as the anti-apoptotic pro-survival PI3K-Akt and Erk 1/2 kinase cascades could open new perspectives for limiting I/R injury associated with LT (Casillas et al., 2006).

Cardiotrophin-1 (CT-1) and alpha-lipoic acid (LA) could be promising drugs against I/R injury associated with LT because their benefits on pro-survival kinase cascades. The pretreatment of isolated hepatocytes with the pro-apoptotic mediator transforming growth factor-beta stimulates CT-1 production. In addition, pretreatment with CT-1 protects rats against fulminant liver failure after subtotal hepatectomy. This protective effect was associated with reduced caspase-3 activity and activation of Erk1/2 and PI3K/Akt pathways (Bustos et al., 2003). Recent research points to the potential of preconditioning with LA for hepatic IRI, which is mediated via the PI3K/Akt pathway. However, neither

Bad nor eNOS phosphorylation was increased after LA pretreatment, suggesting a new mechanism by which LA exerts antinecrotic but not antiapoptotic action during hepatic I/R (Muller et al., 2003). This could be of special interest to protect steatotic liver grafts, given that necrosis rather than apoptosis is the predominant type of cell death in such cases.

The results, based on isolated perfused liver, indicated that the addition of EGF and IGF-I (separately or in combination) to UW reduced hepatic injury and improved function in both liver types. A combination of EGF and IGF-I resulted in hepatic injury and function parameters in both liver types similar to those obtained by EGF and IGF-I separately. EGF increased IGF-I, and both additives up-regulated AKT in both liver types. This was associated with glycogen synthase kinase-3β (GSK3β) inhibition in non-steatotic livers and peroxisome proliferator-activated receptor gamma (PPARγ) over-expression in steatotic livers. The benefits of EGF and IGF-I as additives in UW solution were also clearly seen in the LT model, because the presence of EGF and IGF-I (separately or in combination) in UW solution reduced hepatic injury and improved survival in recipients who underwent transplantation with steatotic and nonsteatotic liver grafts. Thus, EGF and IGF-I may constitute new additives to UW solution in steatotic and nonsteatotic liver preservation, whereas a combination of both seems unnecessary (Zaouali et al., 2010).

6.2 Antiapoptotic strategies

An interesting research in hepatic warm ischemia by Bailly-Maitre et al. has pointed to BAX inhibitor-1 (BI-1) as a regulator of the endoplasmic reticulum (ER) stress-mediated apoptosis pathway (Bailly et al., 2006). The results could lead to new strategies for reducing I/R injury associated with LT. Some mechanisms of ER stress-mediated apoptosis are briefly described below. During liver ischemia, hypoxia-induced ATP deficiency promotes the release of Ca^{2+} from ER to cytosol. The depletion of ER Ca^{2+} stores triggers downstream ER stress pathways that induce apoptosis. The pro-apoptotic Bcl-2 family members BAX and BAK, localized to the ER, also induce emptying of ER Ca^{2+} pools concomitantly with Ca^{2+} translocation into the mitochondria (Breckenridge et al., 2003). In addition, I/R initiates protein misfolding in the ER, which can activate a highly conserved unfolded protein response (UPR) signal transduction pathway. The UPR is characterized by coordinated activation of three ER transmembrane proteins, IRE1, PKR-like ER kinase (PERK) and activating transcription factor (ATF)-6. If the damage is so severe that homeostasis cannot be restored, ER stress signal transduction pathways ultimately initiate apoptosis (Oyadomari & Mori, 2004; Xu et al., 2005). The study by Bailly-Maitre indicated that the ER membrane protein BI-1 protects against apoptosis induced by ER stress. Compared to wild-type BI-1 mice, BI-1 knockout mice subjected to hepatic ischemia/reperfusion exhibited greater elevation in caspase-9 activity, more activation of IRE1, ATF6 and JNK, and greater increases in expression of CHOP and spliced X-box binding protein 1 (XBP-1) (Bailly et al., 2006). Thus, strategies aimed at modulating BI-1 as well as other component of ER stress-mediated apoptosis could protect not only against ER stress but also against the mitochondrial-dependent apoptosis pathway. In liver, the small molecule chemical chaperones, 4-PBA and Tauroursodeoxycholic acid (TUDCA) protect against I/R-induced ER stress-mediated cell death in non-steatotic livers undergoing ischemic conditions (Falasca et al., 2001; Vilatoba et al., 2005). 4-PBA reduced inflammatory response, apoptosis and mortality in non-steatotic livers undergoing total hepatic ischemia (Vilatoba et al., 2005). The addition of TUDCA to UW preservation solution protected non-steatotic livers, specifically sinusoidal lining cells and hepatocytes

against cold ischemia injury (Falasca et al., 2001). Recent studies indicated that PBA, and especially TUDCA, reduced inflammation, apoptosis and necrosis, and improved liver regeneration in both steatotic and non-steatotic livers in partial hepatectomy under vascular occlusion. Both compounds, especially TUDCA, protected both liver types against ER damage, as they reduced the activation of two of the three pathways of UPR (namely inositol-requiring enzyme and PKR-like ER kinase) and their target molecules caspase 12, c-Jun N-terminal kinase and C/EBP homologous protein-10. Only TUDCA, possibly mediated by extracellular signal-regulated kinase upregulation, inactivated glycogen synthase kinase-3β. This in turn, inactivated mitochondrial voltage-dependent anion channel, reduced Cyt c release from the mitochondria and caspase 9 activation and protected both liver types against mitochondrial damage (Ben Mosbah et al., 2010). Also, strategies aimed at modulating component of ER stress-mediated cell death could protect not only against ER stress but also against the mitochondrial-dependent apoptosis pathway. A recent study indicated that TUDCA reduced ER stress in steatotic liver transplantation. Further studies will be required to elucidate whether these chemical chaperones such as 4-PBA and TUDCA could be considered as useful strategies in clinical LT. They have been used for clinical treatment of urea cycle disorders, cholestatic liver diseases and cirrhosis (Ben Mosbah et al., 2010). Results of clinical trials have shown that 4-PBA has few side effects and is safe for patients since it is well tolerated at high dose for long periods of time (Özcan et al., 2006). TUDCA is a derivate of an endogenous bile acid, and it has been safely used as a hepatoprotective agent in humans with cholestatic liver diseases (Falasca et al., 2001).

Recently, autophagy has been described to be activated in stress conditions to ensure cell survival by limiting necrosis or apoptosis *in vivo*. Autophagy is a catabolic pathway triggered following various stress conditions, such as starvation or transient hypoxia, and aimed to restore adequate intracellular ATP and aminoacids levels and to eliminate damaged organelles (Degli et al., 2011). Autophagy has been shown to retard cell death by suppressing ER stress. Thus, the possibility that activation of autophagy may be involved in ER stress attenuation in steatotic livers, and that the modulation of autophagy and ER stress can have beneficial effects in clinical LT should not be discarded.

6.3 Omega-3 PUFAs

Manipulation of the chemical composition of hepatic lipids may evolve as a useful strategy to expand the donor pool and improve the outcome after LT. Macrosteatotic livers disclosed an abnormal omega-6: omega-3 PUFA ratio that correlates with a microcirculatory defect that enhanced reperfusion injury (El-Badry et al., 2007). Therefore, normalization of the Ω-6:Ω-3 FA ratio appears to be crucial for protection of the steatotic liver from reperfusion injury. Preoperative dietary omega-3 PUFAs protect macrosteatotic livers against reperfusion injury and might represent a valuable method to expand the live liver donor pool (El-Badry et al., 2007). Clavien *et al.*, treated three live liver donors with moderate degrees of steatosis by oral administration of X-3 FAs. All donors showed a significant reduction of hepatic fatty infiltration within one month. Subsequently, LT was carried out for three candidates with uneventful outcomes for both donors and recipients. A very promising option to prevent post-transplant complications appears to be the use of a pretreatment with X- 3 FAs. However, the approach is only feasible in living donation since requires oral administration of X-3 FAs before organ procurement (McCormack et al., 2011).

Due to large inconsistencies in the qualitative and quantitative measurement of fat deposits in the liver, new techniques of assessment of steatosis are needed. Computerized programs have been developed to more objectively quantitate hepatic steatosis by determining the area occupied by lipid droplets in a given field of a liver section (El-Badry et al., 2009). However, these quantitative methods provide information only on the total amount of fat, omitting any data on the chemical composition of hepatic lipids. Therefore, novel and objective tools, such as measurement of the X-6 and X-3 FAs and prostanoid levels in liver biopsy samples, may help prediction of the magnitude of reperfusion injury (McCormack et al., 2011).

6.4 Adipocytokines derived from liver and/or adipose tissue

To date, adipose tissue has been considered the major site for endogenous adiponectin production, although there are other potential sources, including the liver (Massip-Salcedo et al., 2008; Neumeier et al., 2006). A recent study indicated that steatotic livers can generate adiponectin as a consequence of I/R (Massip-Salcedo et al., 2008). The role of adiponectin in hepatic I/R injury remains unclear. Adiponectin silent small interfering RNA (siRNA) treatment decreased oxidative stress and hepatic injury in steatotic livers. PPAR-α agonists as well as ischemic preconditioning (PC), through PPAR-α, inhibited mitogen-activated protein kinase expression following I/R. This in turn inhibited the accumulation of adiponectin in steatotic livers and reduced its negative effects on oxidative stress and hepatic injury (Massip-Salcedo et al., 2008). However, another study by Man et al., 2006 in small fatty grafts, adiponectin treatment exerted anti-inflammatory effects that down-regulated TNF-α mRNA and vasoregulatory effects that improved the microcirculation. Adiponectin anti-inflammatory effects also include the activation of cell survival signaling via the phosphorylation of Akt and the stimulation of NO production. Additionally, the studies by Man et al., 2006 showed the anti-obesity and proliferative properties of adiponectin in small fatty transplants. Thus, on the basis of the different results reported to date in hepatic I/R, it is difficult to discern whether we should aim to inhibit adiponectin, or administer adiponectin to protect steatotic livers against cold ischemia associated with transplantation.

Levels of adiponectin are reduced in obese subjects (Bugianesi et al., 2005; Targher et al., 2006; Weyer et al., 2001) and in experimental models of fatty livers, irrespective of the type of steatosis (induced by diet or alcohol) (Rogers et al., 2008; Xu et al., 2003). Indeed, in a cohort of 68 obese individuals, serum levels of adiponectin significantly predicted hepatic steatosis and hepatic damage (Schäffler et al., 2005; Targher et al., 2004). Research aimed at identifying prognostic factors in LT are both necessary and relevant. Further investigations will be required to elucidate whether measurements of adiponectin in serum, a non-invasive tool, might predict the severity of steatosis and liver damage and contribute to the identification of steatotic liver donors with a high risk for transplantation. The decision to implant or reject a steatotic liver is difficult due to the risk of impaired graft function or even failure after implantation. How much fat, and what types of fat, represent a significant risks for primary non-function of the graft remain under debate. The assessment of donor liver fat is a difficult task for the transplant team due to large inconsistencies in the qualitative and quantitative measurement of fat deposits in the liver (El-Badry et al., 2009; McCormack et al., 2011).

Retinol binding protein 4 (RBP4) is an adipokine synthesized by the liver, whose known function is to transport retinol in circulation. However, the role of RBP4 in the liver is largely unknown. A recent study indicated that steatotic liver grafts were found to be more vulnerable to the down-regulation of RBP4 and the over-expression of PPARγ. RBP4 treatment (through AMP-activated protein kinase (AMPK) induction) reduced PPARγ over-expression, thus protecting steatotic liver grafts against I/R injury associated with transplantation. In terms of clinical application, therapies based on RBP4 treatment and PPARγ antagonists might open new avenues for steatotic LT and improve the initial conditions of donor livers with low steatosis that are available for transplantation. (Casillas et al., 2011).

6.5 Surgical strategies

The response of hepatocyte to ischemia never ceases to be surprising. In fact, contrary to what might be expected, the induction of consecutive periods of ischemia to the liver does not provoque an additive effect in terms of the hepatocyte lesion. Murry et al. have reported that ischemic PC based on a brief period of ischemia followed by a short interval of reperfusion prior to a prolonged ischemic stress protects against I/R injury (Murry et al., 1986). The molecular basis for PC consists of a sequence of events: in response to the triggers of PC, a signal must be rapidly generated which is then transduced into an intracellular message leading to the amplification of the effector mechanism of protection (Cutrin et al., 2002; Serafin et al., 2004b). As in the pathophysiology of hepatic I/R, in the modulation of hepatic injury induced by IP there is a complex interaction between different cell types.

The present review is focused on some of the proposed mechanisms leading to the development of hepatocyte resistance to I/R injury following hepatic PC (see Fig. 4). Vasoactive substances such as adenosine, NO, bradykinin, etc, have been considered as the major players in triggering preconditioning (Cutrin et al., 2002). In addition to the extracellular mediators, PC involves activation of intracellular messengers such as PKC, AMPK, p38 MAPK, Ik kinase; signal transducer and activator of transcription-3 (STAT3) and transcription factors including NFκB and heat shock transcription factor 1 (HSF1) (Carini & Albano, 2003; Selzner, 2003) (see Fig. 4). The downstream consequences of these pathways could be cytoprotective by abrogation of cell death pathways, stimulating antioxidant and other cellular protective mechanisms including MnSOD and heat shock proteins (HSPs), and by initiating entry into the cell cycle (Cutrin et al., 2002; Selzner, 2003). The benefits of PC on energy metabolism, inflammatory mediators including ROS and TNF, mitochondrial dysfunction, KC activation, and microcirculatory disorders associated with I/R injury have also been described (Casillas et al., 2006; Massip-Salcedo et al., 2007). PC via AMPK activation, reduced the ATP depletion thus attenuating the accumulation of glycolytic intermediates and lactate production during hepatic sustained ischemia (Peralta et al., 2000b). The benefits of PC on oxidative stress could be explained by the induction of antioxidants, such as SOD and HSPs as well as by its effect on XDH/XOD (Carini & Albano, 2003; Casillas et al., 2006; Massip-Salcedo et al., 2007). PC reduced the accumulation of xanthine during ischemia and prevented the conversion of XDH to XOD, thus preventing the deleterious effect of this ROS generating system on liver (Fernández et al., 2002; Serafin et al., 2004b) (see Fig. 4). It is possible that NFkB and p38 MAPK-regulated transcription factors (ATF-2 and MEF2C) might be responsible for inducing the expression of protective

genes, including SOD. HSPs induced by PC might contribute to improve membrane potential and respiratory control in hepatic mitochondria, allowing a faster recovery of ATP on reoxygenation (Carini & Albano, 2003; Massip-Salcedo et al., 2007). The modulation of inflammatory response by hepatic PC has been also reported in different experimental models of warm and cold hepatic ischemia. PC reduces neutrophil accumulation, the generation of different cytokines and interleukins including TNF and IL-1 (Casillas et al., 2006; Cutrin et al., 2002; Massip-Salcedo et al., 2007). The benefits of PC were also observed on hepatic microcirculation by inhibiting the effects of different vasoconstrictor mediators such as ETs, thus ameliorating sinusoidal perfusion and microvascular dysfunction (Peralta et al., 1996; Peralta et al., 1999a). The benefits of PC regulating Ang II and adipocytokines such as adiponectin and RBP4 have been also reported in hepatic I/R. PC, through PPARα inhibits adiponectin accumulation in steatotic livers and adiponectin-worsening effects on oxidative stress and hepatic injury in hepatic resactions (Massip-Salcedo et al., 2008). In liver transplantation PC, which increases RBP4 levels, reduced PPARγ levels and hepatic injury in steatotic livers (Casillas et al., 2011). As ER stress activates an adaptive response to injury, modulating ER stress before transplantation by PC could improve the grafted organ viability (see Fig. 4). Along these lines, it has been proposed that induction of ER chaperones, particularly of BiP, underlies the phenomenon of PC in the heart, in which exposure to a transient episode of brief ischemia provides subsequent protection from a

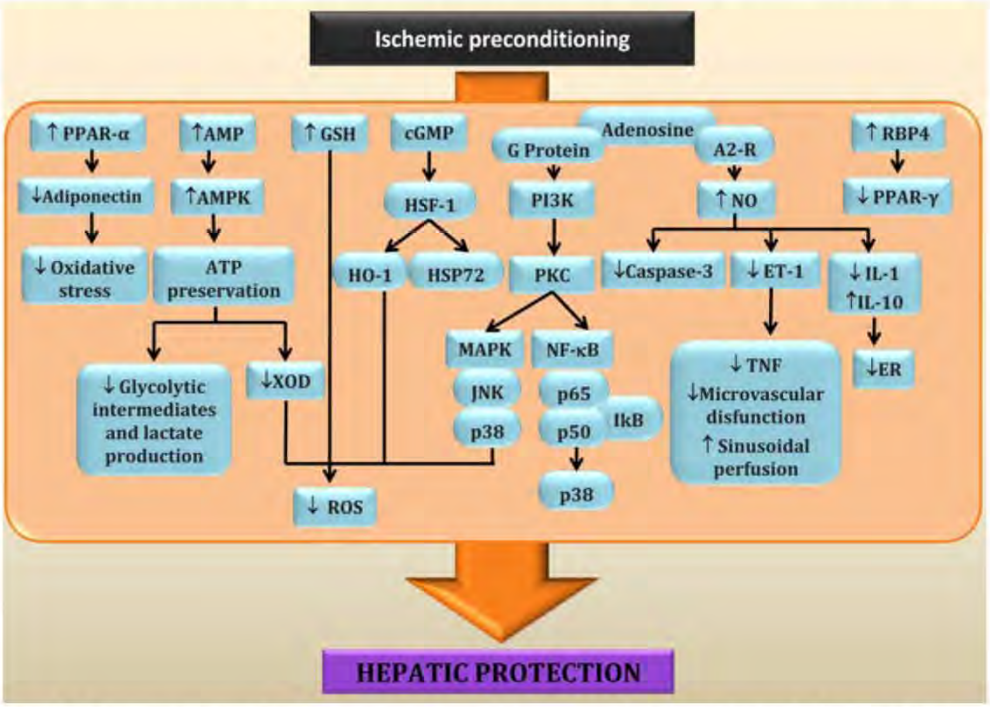

Fig. 4. Molecular basis of the ischemic preconditioning protection. (Carini & Albano, 2003; Casillas et al., 2006; Cutrin et al., 2002; Fernández et al., 2002; Massip-Salcedo et al., 2007, 2008; Peralta et al., 1996, 1999a, 2000b; Selzner, 2003; Serafin et al., 2004b)

sustained ischemic challenge (Kim et al., 2008). It is tempting to speculate that PC activates the UPR, particularly the adaptive and pro-survival aspects of ER stress (Pallet et al., 2009).

Since the effectiveness of PC was first described, numerous efforts have been made to find strategies capable of mimicking its beneficial effects. One of these strategies is known as heat shock preconditioning, in which the organ or the whole body is temporarily exposed to hyperthermia prior to hepatic ischemia. Chemical preconditioning with either doxorubicine, atrial natriuretic peptide or oxidants decreases hepatic injury in several experimental models of I/R. However, their possible clinical application seems limited owing to difficulties in implementing them in clinical practice, toxicity problems and the side-effects that have been identified (Casillas et al., 2006; Massip-Salcedo et al., 2007; Peralta et al., 1999a).

The benefits of PC observed in experimental models of hepatic warm and cold ischemia created the need for human trials of PC. To date, PC has been successfully applied in human liver resections in both steatotic and non-steatotic livers. The effectiveness of PC in hepatic surgery was first reported by Clavien et al., 2003, but unfortunately, in this study, it proved ineffective in elderly patients. It is well known that the impact of cold ischemia on organ function becomes even more significant as the age of the donor increases (Busuttil & Tanaka, 2003). Recent research indicates that melatonin prevents oxidative stress and inflammatory response in hepatocytes from elderly rats and this could improve the viability of liver grafts from elderly donors and increase the effectiveness of PC (Castillo et al., 2005).

Prevention of post-hepatectomy liver insufficiency by PC, particularly in patients with cirrhotic or steatotic livers has also been demonstrated (Nuzzo et al., 2004). A clinical study by Koneru and colleagues showed no effects of PC on cadaveric donor livers compared with controls. However, the study consisted of clamping the hepatic vessels for a period of 5 min, and as the authors concluded, that may be insufficient to obtain a beneficial effect from PC (Koneru et al., 2005). Another clinical study carried out by Azoulay and colleagues using the model of cadaveric whole liver transplantation showed that PC based on 10 min of ischemia was associated with better tolerance to ischemia. However, this was at the price of decreased early function (Azoulay et al., 2005). Beginning this year, Jassem and colleagues concluded that 10 min of preconditioning was effective to protect cadaveric donor allografts from cold ischemia, reduced inflammatory response and resulted in better graft function (Jassem et al., 2006). Further randomised clinical studies are necessary to confirm whether PC is appropriate for LT in clinical practice. The potential applications of PC in human LT are numerous. PC also has the potential to increase the number of organs suitable for LT since it can improve the outcome for marginal grafts that would not otherwise have been transplanted. Its benefits to reduce the vulnerability of steatotic grafts to I/R injury have also been reported in different experimental studies of LT (Carrasco et al., 2005; Fernández et al., 2004). Interestingly, the effectiveness of PC in clinical practice in major liver hepatectomy opens up new possibilities in living donor liver transplantation, since the ischemia period is similar in both surgical procedures. Moreover, PC increases liver regeneration, the most critical aspect to be considered in living donor liver transplantation (Franco et al., 2004). Again, PC may also have a role in the transplantation of small grafts whose pathophysiology overlaps with I/R injury. In fact, a study published by Barrier et al., 2005 has shown the benefits of PC in transplantation from living human liver donors. PC is easy to apply, inexpensive and does not require the use of drugs with potential side effects.

One disadvantage of PC is that it requires a period of pre-ischemic manipulation for organ protection.

7. Conclusion and perspectives

The hope of finding new surgical and pharmacological therapeutic applications provides a strong impetus to identify the mechanisms responsible for the failure of fatty livers. We must continue conducting researches attempting to improve the outcomes of LT using fatty liver grafts. Before a complete definition of a successful therapeutic strategy based on regulating hepatic I/R injury is stated, several additional points need to be addressed. The effects of the new potential protective strategies (TMZ, AICAR, RAS modulators, PI3K and ERK1/2 modulators, anti-apoptotic strategies, omega-3 PUFA, adiponectin, RBP4 and PC) on the pathways involved in the inflammatory process and lipid metabolism have only just been mapped. The success of these protective strategies might depend on the surgical procedure. Moreover, the response of different type of liver to these treatments might differ and involve different signal transduction pathways that are at present marginally understood. Whether the above-mentioned approaches can be translated into as viable options in the clinical practice remain unknown, but further researches are required to optimize the their use (e.g. dose, pharmacokinetics…etc). Such approaches have the potential to increase the number of organs suitable for transplantation, since they may improve the outcomes of marginal grafts that would not otherwise have been used.

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Elias-Miró M. and Jiménez-Castro M.B. contributed equally to this work

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Chapter 4

ADENOSINE TRIPHOSPHATE IN EXPERIMENTAL LIVER SURGERY

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ABSTRACT

The shortage of organs has led centers to the acceptance of marginal grafts such as fatty livers, small-for-size liver or aged donors. However, the clinical problem is unresolved since this type of liver tolerates poorly hepatic ischemia-reperfusion (I/R) and show regenerative failure after liver surgery. The use of marginal liver for transplant is associated with increased risk of primarily non-function or dysfunction after surgery, being the deficiencies in energy metabolism one of the main mechanisms responsible for the vulnerability of this liver type to I/R injury and regenerative failure. Indeed, experimental studies and clinical observations clearly indicate that marginal livers show more adenosine triphosphate (ATP) depletion during ischemia and synthesize less ATP than normal livers during the early phase of reperfusion. This book chapter will be focused on the role of ATP in hepatic I/R injury and the mechanisms responsible of ATP depletion in both marginal and normal livers. We will show that the deleterious effects of ischemia on ATP depletion and the lactate production limit survival of hepatocytes, being this effect more exacerbated in marginal livers. Also, we will explain how different conditions, including the presence of fatty infiltration or starvation, affect ATP recovery during reperfusion, a prerequisite for liver graft viability after surgery. In hepatic I/R injury cell death can occur via necrosis or apoptosis. We will review the key role of ATP as a putative apoptosis/necrosis switch: when ATP depletion is severe, necrosis ensues before the activation of the energy-requiring apoptotic pathway. The present book chapter will discuss how ATP depletion and its posterior restoration depends on the type of ischemia (cold or warm ischemia), the type of liver, duration and the extent of hepatic ischemia, starvation, and the presence of liver regeneration. We will show that the mechanism responsible for ATP recovery during reperfusion depends of the experimental model used. Therefore, is very important to choose to standardize experimental conditions according to the clinical question being

answered. As the decrease in ATP immediately after partial hepatectomy associated with reduced-size liver transplantation trigger a signal to activate the catabolism of existing peripheral adipose stores; we will review how lipid accumulation is used for ATP synthesis, necessary for liver regeneration. Finally, we will consider pharmacological and surgical strategies that prevent ATP degradation and/or increase ATP restoration during reperfusion as this may improve the post-transplant outcomes and could reduce waiting list for liver transplantation.

INTRODUCTION

Hepatic ischemia/reperfusion (I/R) injury during hepatectomy and liver transplantation (LT) is a major cause of liver dysfunction. LT has evolved as the therapy of choice for patients with end-stage liver disease. However, the waiting list for liver transplantation is growing at a rapid pace, whereas the number of available organs is not increasing proportionately. The potential use of marginal liver for transplantation has become a major focus of investigation. However, marginal livers are more susceptible to I/R injury. Indeed, the use of steatotic livers for transplantation is associated with an increased risk of primary nonfunction or dysfunction after surgery. In addition, the occurrence of postoperative liver failure after hepatic resection in a steatotic liver exposed to normothermic ischemia has been reported. Therefore, minimizing the adverse effects of I/R injury could improve outcomes in liver surgery, increase the number both of suitable transplantation grafts and of patients who successfully recover from liver transplantation. In spite of ongoing intensive research efforts, only a few protective strategies are currently available [1].

CHARACTERISTICS OF ATP IN DAMAGE AND REGENERATIVE FAILURE IN HEPATIC I/R

The stresses of hypoxia, ischemia and exposure to toxic chemicals cause cells to lose viability. Typically, cell death occurs within a few hours, sometimes within minutes. Onset of cell death is the consequence of acute and profound disruption of cellular metabolism, leading most often to ATP depletion, ion dysregulation, cellular swelling, and activation of degradative enzymes. ATP degradation during ischemia also leads to an acceleration of glycolysis, resulting in the net formation of lactate [2, 3]. We and others [4, 5, 6] suggest that when glycolysis is the only source of ATP, such as during anoxia, changes in adenine nucleotides such as decrease in ATP or increase in AMP probably play the main regulatory role in the control of glycolytic pathway, at least after the first minutes of ischemia. Restoration of blood supply to an organ after a critical period of ischemia results in parenchymal injury and dysfunction of the organ referred to as reperfusion injury. The critical ischemia period is dependent on the organ being of 15–20 min [7] in the liver. Reperfusion following periods exceeding the critical ischemia period results in endothelial and parenchymal injury. Ischemia causes anoxia, acidosis from anaerobic metabolism and ATP depletion. Restoration of blood supply results in reactive oxygen species (ROS) generation, causing oxidative stress. The mitochondria are the principal source of ATP. Mitochondria have a distinctive two membrane construction. The inner membrane is impermeable to even

the smallest of ions, the hydrogen ion. Thus, metabolite exchange across the inner membrane must occur via specific transporters and exchangers, such as the adenine nucleotide transporter (ANT), which exchanges ATP for adenosine diphosphate (ADP); the phosphate transporter (PT), which exchanges Pi for OH–; and carboxylate transporters (CT) and carnitine shuttle (CS), responsible for taking up various respiratory substrates. With the major exception of oxygen, which is soluble in lipid bilayers, these and related transporters catalyze the uptake and release of the products and reactants of oxidative phosphorylation. The ATP synthesis itself occurs on the inner surface of the inner membrane driven by an electrochemical gradient of protons (mostly consisting of membrane potential) created by proton-pumping respiratory complexes embedded in the inner membrane [8]. The mitochondrial damage caused by oxidative stress could result in reduced ATP production and compromised cell function [9]. After hepatic ischemia and reperfusion injury, vascular NTPDase activity is lost and deletion of NTPDase1 leads to significantly increased injury and decreased survival. Furthermore, post-transplant biochemical activity of NTPDase1 is lost initially postoperatively and is reestablished within days to weeks later in the surviving, potentially accommodated grafts [10].

Extracellular nucleotides and nucleosides are released in a regulated manner from cells by a variety of mechanisms. Such pathways include exocytosis of ATP/UTP-containing vesicles, facilitated diffusion via connexin-43 hemichannels, by putative ABC transporters or potentially by poorly understood electrodiffusional movements through ATP/nucleotide channels [11-18]. Under pathophysiological conditions, the release of nucleotides and the expression of purinergic receptors is increased markedly in injured or stressed cells [14]. Extracellular ATP entering the liver by the portal vein is rapidly metabolized after a single passage through the liver [19]. Therefore, metabolic stimulation by extracellular nucleotides might be expected to be more pronounced in the periportal region. In addition, ATP can be released by hepatocytes into different extracellular compartments: via basolateral, sinusoidal or apical exocrine routes. Secretion of ATP into the bile is mediated by an increase in cholangiocyte cell volume, which stimulates nucleotide release by vesicular exocytosis [20, 21]. Canalicular nucleotide and nucleoside levels are further regulated and controlled by the presence of a canalicular Na+-dependent nucleoside transporter that removes adenosine from the bile [22]. Salvage of nucleotides in bile may be crucial in the maintenance of appropriate nucleotide/nucleoside concentrations within hepatocytes or within the entire organism [23].

It should be considered that the decrease in ATP in non-steatotic and steatotic liver grafts immediately after transplantation might represent a signal for liver regeneration. Although cytokines and growth factors are known to be involved in the multi-step process of liver regeneration, other endocrine, paracrine and neural factors may also play important roles especially in early stages, including extracellular ATP which has been less studied [24, 25]. Decline in ATP after PH was not caused by either increased energy demand, mitochondrial damage or uncoupling of oxidative phosphorylation [26, 27]. Shear stress is known to release ATP from isolated hepatocytes and the increased blood flow throught the remant liver after PH may have a similar effect. ATP is released from liver immediately after PH, concomitantly with an acute portal hyperpressure in LRLT. A rapid release of ATP after PH could function as a paracrine signal that is recognized by purinergic receptors on neighboring cells that contribute to the priming phase during the onset of regeneration. Lysosome fusion with plasma membrane constitutes a form of regulated exocytosis that can allow ATP release after PH [24-29].

ATP RECEPTOR AND SIGNALING PATHWAYS POTENTIALLY INVOLVED IN HEPATIC I/R

Over fifteen P2-receptors of different specificities transmit signals from extracellular nucleotides, triggering and modulating vascular and immune cell activation processes, metabolism, nitric oxide (NO) release, adhesion, migration, proliferation and apoptosis [30, 31]. Since the original cloning of the P2Y2R subtype, at least eight P2YR and seven P2XR subtypes have been cloned and functionally identified. There are two main families of nucleotide receptors: P2X are "rapid" ligand-gated ion channels permeable for Na+, K+ and also Ca2+ (subtypes P2X1-7) [32] and P2Y are the "slow" metabotropic receptors (P2Y1, 2, 4, 6, 11–14). P2Y receptors are 7-transmembrane Gq- or Gi-protein linked and initiate signal transduction coupled to activation of phospholipase C, or to inhibition of adenylate cyclase, respectively [33]. P2X and P2Y purinergic receptors are expressed by hepatocytes [34, 35]. Only the P2Y1, P2Y2 and P2Y13 receptors appear to be of functional relevance for the hepatocyte [36, 37]. Depending upon the repertoire of receptors and signaling components, P2R influence cellular activation, proliferation and the induction of apoptosis. For example, in the vascular system, extracellular nucleotides and nucleosides can influence platelet activation, thrombosis, inflammatory processes, and vasomotor responses, [38-41]. ATP and ADP appear to regulate hemostasis through the activation of platelet P2 receptors. Platelet P2Y12 is perhaps the best known purinergic receptor [42]. ATP and UTP also stimulate endothelial P2Y receptors to release prostacyclin (PGI2) and NO; two vasodilators and inhibitors of platelet aggregation [43-47]. This latter protective action of ATP may limit the extent of intravascular platelet aggregation and to help localize thrombus formation to areas of vascular damage [45, 48, 49]. The receptors P2Y1 and P2Y2 on vascular endothelial cells are also important receptors in the mediation of vascular inflammation [50-52]. In addition, ATP also stimulates P2X receptors to cause plasma membrane permeabilization, induction of apoptosis, organic anion transport, and stimulation of Ca2+ mobilization [39, 53]. The major effect of P2X7 receptors is the induction of apoptosis [54, 55]. Hepatocytes and bile ductular cells have been shown to interact and communicate via ATP release in a paracrine manner and nucleotides may be involved in the regulation of canalicular contraction and bile secretion. The binding of nucleotides to apical P2Y2-receptors may facilitate the coordination of bile formation as a consequence of the paracrine hepatobiliary coupling [56]. Functional expression profiles of P2Y1 and P2Y2 receptors have been described for the hepatic artery and portal vein [57, 58]. In response to nucleotides, sinusoidal endothelial cells secrete Prostaglandin E2 (PGE2) in a P2Y dependent manner [59]. Some P2Y receptors undergo agonist-induced desensitization [50]. Given previous studies, rapidly desensitizing receptors and/or channels (P2Y1, P2Y2, P2X1 and P2X3) contrast with slow desensitizing receptors such as the P2Y6, P2X2 and P2X7 receptors (the last has proven to be very important in inflammatory reactions); P2X4 is intermediate in this regard [60].

ECTONUCLEOTIDASES AND SIGNALING PATHWAYS POTENTIALLY INVOLVED IN HEPATIC I/R

Ectonucleotidases consist of families of nucleotide metabolizing enzymes that are expressed on the plasma membrane and have externally orientated active sites (Figure). The major ectonucleotidases activity of hepatocytes lies within the bile canalicular domain. These ectoenzymes operate in concert or consecutively and metabolize nucleotides to the respective nucleoside analogs. They have the potential to decrease extracellular concentrations of nucleotides and to generate nucleosides. The relative contribution of the distinct enzymes to the modulation of purinergic signaling may depend on the availability and preference of substrates and on cell and tissue distribution. Ectonucleotidases modulate P2-receptor-mediated signaling. Alterations in extracellular nucleotide levels can increase or decrease P2 receptor activity or lead to P2 receptor desensitization [61]. Furthermore, generation of extracellular adenosine not only abrogates nucleotide-mediated effects but also activates adenosine receptors, often with opposing (patho-) physiological effects. Ectonucleotidases also produce the key molecules for purine salvage and consequent replenishment of ATP stores within multiple cell types [62]. Indeed, although nucleotides do not appear to be taken up by cells, dephosphorylated nucleoside derivatives interact with several specific transporters to enable intracellular uptake via membrane passage [23, 63]. There are four major families of ectonucleotidases in the liver as described below.

3.4.1. Ecto-nucleoside triphosphate diphosphohydrolases (NTPDases of the CD39 family)—It is now known that there are three ectonucleotidases largely responsible for the bulk of ATPase activity in the liver: NTPDase1, which is present on Kupffer and vascular endothelial cells, NTPDase2, which is expressed by portal fibroblasts and activated hepatic stellate cells and NTPDase8 that seems to be the major ATPase of the hepatic canaliculus. Other hepatic NTPDases such as NTPDase3 on stellate cells and NTPDase5 have been described on liver cells, however, functional data is still lacking. Quiescent sinusoidal endothelial cells do not express CD39. However, under specific conditions of activation e.g. proliferation after partial hepatectomy, expression of CD39 on sinusoidal endothelial cells is notably upregulated and is of substantial functional relevance.

3.4.2. Nucleotide pyrophosphatase/phosphodiesterases (NPP)—NPP hydrolyze pyrophosphate or phosphodiester bonds of nucleotides, metabolizing ATP to AMP and diphosphates. The family of NPPs consists of seven members (NPP1-7). NPP1 is expressed in hepatocytes and localizes basolateral membrane [64-66]. The expression of NPP1 is associated by hepatocellular growth. It decreased during liver regeneration following partial hepatectomy [67]. This observation underscores the relevance of ATP for hepatocellular proliferation [68]. NPP3 is the major NPP isoenzyme at the apical membrane of hepatocytes [65, 66].

3.4.3. Alkaline phosphatases (ALP)-Alkaline phosphatases are hydrolases responsible for removing phosphate groups in the 5- and 3- positions from many types of molecules, including nucleotides, proteins, and alkaloids [69].

3.4.4. Ecto-5'-nucleotidase/CD73-Ecto-5'-nucleotidase (CD73; EC 3.1.3.5) terminates the dephosphorylation cascade of nucleotides to adenosine [70]. This enzyme has been detected in the canalicular plasma membrane, in the connective tissue of the portal triads and

central veins, and HSC [23,71]. Ecto-5'-nucleotidase is co-expressed with NTPDase8 on the canalicular membrane, thereby regulating biliary nucleotide and nucleoside levels [72].

ROLE OF ATP IN CELL DEATH IN HEPATIC I/R

Cell death typically follows one of two patterns: oncotic necrosis and apoptosis [73]. Apoptosis and necrosis are usually considered separate entities, but an alternate view is emerging that apoptosis and necrosis are frequently the consequence of the same initiating factors and signaling pathways. Rather than being separate entities, apoptosis and necrosis in their pure form may represent extremes on a continuum of cell death [73]. The intracellular ATP levels appear to play a role as a putative apoptosis/necrosis switch: when ATP depletion is severe, necrosis ensues before the activation of the energy-requiring apoptotic pathway.

Necrosis is typically the consequence of acute metabolic perturbation with ATP depletion as occurs in ischemia/reperfusion [73]. Oncotic necrosis is most often the consequence of metabolic injury leading to ATP depletion. As its name implies, swelling is a prominent feature of oncotic necrosis. Early after ATP depletion to hepatocytes, moderate cellular swelling occurs associated with small protrusions of the plasma membrane, called blebs. Bleb formation is likely a consequence of ATP depeletion-dependent cytoskeletal alterations. After many minutes or even hours, a metastable state develops characterized by mitochondrial depolarization, lysosomal breakdown, rapid ion changes and accelerated bleb formation and swelling [74-77]. This metastable state culminates in outright rupture of plasma membrane bleb [76, 77].

Apoptosis represents the execution of an ATP dependent death program, which leads to a caspase activation cascade. Whereas in necrosis large groups of contiguous cells die, in apoptosis individual dying cells separate from their neighbors (See figure 1). Distinctive nuclear changes also occur in apoptosis, including chromatin condensation, internucleosomal DNA degradation, and nuclear lobulation and fragmentation. Eventually, cells fragment into apoptotic bodies that are phagocytosed by adjacent cells and macrophages for lysosomal degradation [73].

A common event leading to both apoptosis and necrosis is mitochondrial permeabilization and dysfunction, although the mechanistic basis of mitochondrial injury may vary in different settings [73]. The mechanisms that induce the release of mitochondrial intermembrane proteins such as cytochrome c remain controversial [78]. In hepatocytes TNFα-and Fas-dependent signalling induces the onset of the mitochondrial permeability transition (MPT). The MPT occurs from the opening of a pore in the inner membrane, the permeability transition pore. MPT leads to large-amplitude mitochondrial swelling, rupture of the outer membrane, and release of cytochrome c and other proteins from the intermembrane mitochondrial space [78]. Other mechanisms for cytochrome c release also seem to exist. In some models, tBid interaction with either Bax or Bak, forms channels in the mitochondrial outer membrane that release cytochrome c and other proteins from the intermembrane space [79, 80]. If MPT onset occurs in relatively few mitochondria, the organelles become sequestered into autophagosomes for lysosomal digestion, a process that eliminates the damaged and potentially toxic mitochondria [78, 81]. When the MPT involves more mitochondria, mitochondrial swelling leads to outer membrane rupture and cytochrome c

release, which activate downstream caspases and other executioner enzymes of apoptosis. When MPT onset is abrupt and involves most mitochondria, ATP becomes profoundly depleted, which blocks caspase activation. Instead, ATP depletion culminates with plasma membrane rupture and the onset of necrotic cell death [73, 82, 83]. Thus, ATP depletion after reperfusion promotes necrotic cell killing while simultaneously suppressing apoptotic signaling. By contrast, if ATP levels are partially restored after reperfusion, necrosis is prevented. During ischemia, the anti-apoptotic Xlinked inhibitor of apoptosis protein (XIAP) decreases progressively in hepatocytes [84]. XIAP antagonizes Cyt c-dependent caspase 9/3 activation [85, 86], and XIAP depletion during ischemia is associated with increased caspase activation and apoptosis after reperfusion. Hepatocytes from XIAP deficient mice show a 10-fold enhancement of apoptosis after short periods of ischemia, which is reverted by treatment with an XIAP expressing adenovirus. Thus, decreases of XIAP during ischemia sensitize hepatocytes to apoptosis after reperfusion [84].

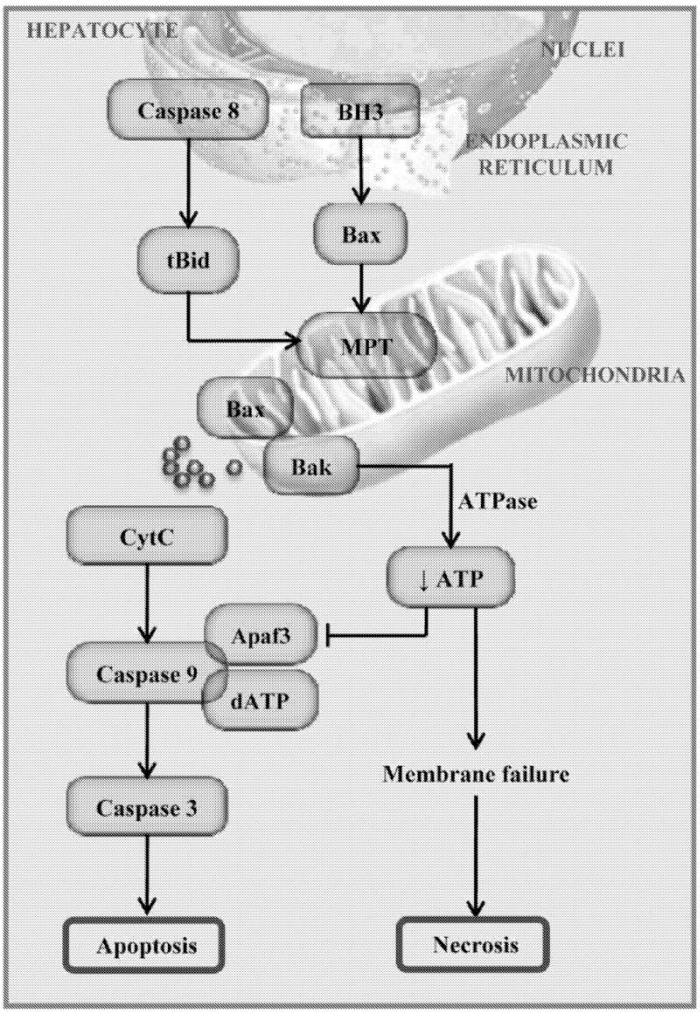

Figure 1. Necrosis and apoptosis in hepatic I/R. Activation of intrinsic and extrinsic pathways converge on mitochondria to induce membrane permeabilization. CytC, cytochrome C; MPT, mitochondrial permeability transition.

ROLE OF ATP IN MARGINAL LIVERS UNDERGOING I/R

Impaired mitochondrial function has been described in fatty livers. Thus, increased susceptibility to mitochondrial dysfunction and consequent ATP depletion, may be a condition that potentiates I/R damage in fatty livers [1]. Experimental studies and clinical observations clearly indicate that fatty livers are capable of synthesizing less ATP than normal livers during the early phase of reperfusion following either warm or cold ischemia [87-89].

The mitochondrial oxidative alterations in fatty liver are associated with an important reduction in the content of the enzyme F0F1-ATP synthase, which can explain the reduced hepatic ATP content seen in these liver types. Moreover, starvation exacerbates the above mentioned alterations to a greater extent in fatty than normal livers. The characterization of the mitochondrial dysfunction is, therefore, of great importance to define new therapeutic approaches in the clinical setting of partial hepatectomy and liver transplantation [90].

Steatosis alters the lipid composition and the fluidity of the mitochondrial membranes and facilitates the formation of lipid peroxidation products. In fatty liver, mitochondrial lipids are likely to be less protected from the attack of free radicals generated both inside and outside the organelle because of the low level of a-tocopherol found in the extra-mitochondrial space and the low GSH content in the intra-mitochondrial compartment. Protein damage from oxidative stress may occur either directly or as a result of lipid peroxidation. An increased level of oxidized products of mitochondrial lipids and proteins could affect some membrane functions, including the integrity of the F0F1–ATP synthase complex. In addition, in fatty livers, glycolytic or other alternative pathways of ATP generation may be somehow impaired or even blocked [90].

In steatotic liver grafts subjected to 6 hours of cold ischemia, necrosis was the predominant process of cell death, and no signs of apoptosis were found. Because apoptosis is an energy-requiring process, the impaired maintenance of ATP levels observed after reperfusion in steatotic livers subjected to long periods of cold ischemia may be linked to a failure to induce apoptosis. Not surprisingly, previously reported data indicate that necrosis rather than apoptosis is the predominant process by which fatty livers undergo cell death. However, Man et al. reported that small steatotic liver grafts subjected to 40 minutes of cold ischemia underwent apoptosis. Because of the short cold ischemia times, the ATP depletion may not have been sufficiently severe to induce necrosis, and this may have allowed apoptosis to take place [24].

The lower ATP and adenine nucleotide levels in steatotic livers preserved in University of Wisconsin solution could be caused by mitochondrial damage. Alterations in oxidative phosphorylation during preservation are greatly enhanced by fatty infiltration due to damage to respiratory chain complex. Other studies have shown that during warm ischemia or transplantation, the level of mitochondrial uncoupling protein 2 (UCP2) is 4 to 5 times higher in steatotic livers versus nonsteatotic livers. This finding has been associated with a reduced ability to synthesize ATP upon reperfusion. Previous studies have indicated that steatotic livers have a reduced ability to respond to endoplasmic reticulum (ER) stress [91]. ER stress in steatotic livers activates the mitochondrial cell death pathway, which results in inflammation, apoptosis, and necrosis. Man et al. found that UCP2 and fatty acid synthase (FAS) were activated in small fatty liver grafts after transplantation, and they promoted ATP

depletion and necrosis. It is well known that PPARα affects the transcription of a number of genes involved in lipid turnover and peroxisomal and mitochondrial β-oxidation, resulting in the generation of ATP. In conditions in which PPARα function and/or expression is altered such as hepatic steatosis, and small-size liver grafts, FA metabolism is deviated toward the accumulation of inadequately metabolized fat, favoring ROS generation. Consequently, ATP production is decreased, and the demise of hepatocytes via necrotic cell death is increased, halting liver repair [91].

Steatotic livers impaired ATP synthesis caused by intracellular accumulation of nonesterified fatty acids that increase mitochondrial uncoupling and inhibit gluconeogenesis [92-94]. Upon I/R, mitochondria isolated from fatty livers was associated to decreased mitochondrial transmembrane potential (DW) and oxygen consumption, as well as decreased efficiency of the phosphorylation system, caused by depletion of the adenine nucleotide translocator [95, 96]. The phosphorylative efficiency was affected in fatty livers upon I/R. Such alteration caused a decrease in the ATP/ADP ratio. The decrease in ATPase activity in fatty livers is the probable cause for the loss of mitochondrial phosphorylative efficiency induced by I/R [1].

Aging is associated with a variety of decreased mitochondrial function in liver [97]. In isolated rat hepatocytes age is associated with an alteration in mitochondria membrane potential, along with an increase in the size of mitochondria. Older livers have a higher susceptibility to normothermic ischemic injury as a result of mitochondrial dysfunction. Le Couteur et al described a decrease of sinusoidal fenestration and an increased deposition of collagen in old rats. This might contribute to the decrease in ATP/ADP ratio observed in rats with advanced age [97].

THERAPEUTIC STRATEGIES BASED ON THE REGULATION OF ATP IN HEPATIC I/R

Surgical Strategies

To limit injury, interventions that prevent lactate production and/or loss of high-energy metabolites such as ATP might be effective. Previous studies indicated that PC, consisting of a brief stress period induced by short periods of I/R, is able to induce the activation of AMPK before the sustained ischemia. In the liver, the PC effect has been demonstrated in animal models, as well as clinically during hemihepatectomies and in deceased donors [98-100]. This surgical strategy preserved to a greater extent ATP, adenine nucleotide pool, and adenylate energy charge; the accumulation of adenine nucleosides and bases was much lower in preconditioned livers, thus reflecting slower adenine nucleotide degradation. This process may explain the beneficial effect of PC on energy metabolism, reflected in lower ATP degradation and lactate accumulation during prolonged ischemia and reduced hepatic reperfusion injury [101]. Results obtained in normothermic conditions suggest that ATP preservation induced by PC may be central to the protection of steatotic livers against the I/R injury [101].

In the liver, ATP preservation does not seem to be related to ATP production via anaerobic glycolysis because there is a close inverse relationship between ATP and glycolytic

activity, as estimated by lactate production. Consequently, ATP preservation probably results from decreased ATP utilization. For instance, this could also explain the slower ATP degradation in preconditioned livers [102].

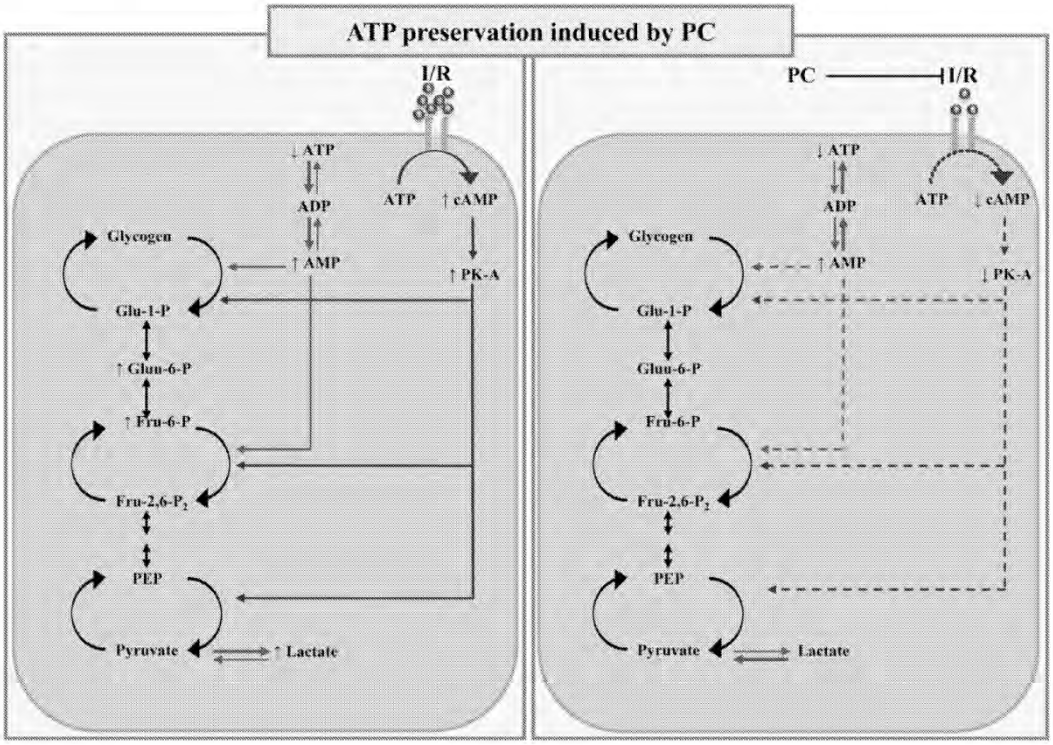

Figure 2. Schematic representation showing the proposed mechanisms by which PC protects from hepatic I/R injury. Preconditioning reduces ATP degradation and the increase in AMP and cAMP, thus attenuating the accumulation of glycolytic intermediates and lactate production during sustained ischemia. ATP, adenotine triphosphate; ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; Fru-1,6-P2, fructose 1,6-biphosphate; Fru-6-P, fructose 6-phosphate; Glu-1-P, glucose 1-phosphate; Glu-6-Pase, glucose 6-phosphatase; PEP, phosphoenolpyruvate; PK-A, protein kinase A.

The reduced ATP degradation induced by PC, resulting probably from decreased ATP utilization, would attenuate the net formation of lactate (See figure 2). This effect could be mediated by the action of PC on regulatory metabolites such as cAMP and AMP. Thus the reduced cAMP levels induced by PC could attenuate the modification of key enzymes of the glycogenolytic and glycolytic pathway caused by cAMP-dependent protein kinase. This, in addition to the reduced AMP levels induced by PC, could reduce the activity of key enzymes and the availability of intermediates for anaerobic glycolysis, thus attenuating the glycolytic rate, decreasing the accumulation of acid waste products, and allowing cells to survive in these adverse conditions. The steps modified by PC are shown in Fig. 2. The results point to the induction of metabolic arrest and/or associated metabolic down-regulation as the energetic cost saving mechanisms that could be induced by PC [102]. In steatotic liver transplantation, the reduction in cAMP levels induced by PC protected steatotic livers against hepatic I/R injury. This blockade of cAMP by a mechanism that is independent of NO preserved more of the ATP and adenine nucleotide pool throughout cold ischemia period. This ATP preservation

does not seem to be related to ATP production via anaerobic glycolysis because there is a close inverse relationship between ATP and glycolytic activity (as estimated by the accumulation of hexose 6-phosphates and the production of lactate). Consequently, ATP preservation induced by PC throughout cold ischemia may result from decreased ATP utilization. This could also explain the slower degradation of ATP in the liver that is induced by PC [103]. This was not the case for non-steatotic liver transplantation. Indeed, in non-steatotic liver grafts, cAMP is not involved in the underlying protective mechanism of PC [103].

The effectiveness of PC on mitochondrial dysfunction associated with hepatic I/R have been also reported. In preconditioned fatty livers subjected to I/R, mitochondrial function was improved to values comparable to lean animals subjected to I/R. DW analysis revealed that the capability of creating and maintaining a potential was compromised on mitochondria from fatty livers, a pre-existing condition that was aggravated by I/R. This could be the result of increased permeability of the mitochondrial inner membrane to protons. In fact, the resting mitochondrial oxygen consumption, the state 4 respiratory rate, was also significantly increased in fatty livers upon I/R, in relation to lean and non-I/R fatty animals. PC modulated MPT and prevented the increase in state 4 respiration thus enabling the generation of higher DW [1].

Pharmacological Strategies

The addition of TMZ to UW solution reduced mitochondrial damage, increased ATP levels and reduced hepatic injury and ameliorated hepatic functionality in both types of the liver [105]. Liver transplantation may benefit from strategies such as the addition of TMZ to the preservation solution as this seems to help maintain appropriate bile duct cell functions. It is well known that poor recovery after ATP depletion appear to contribute to bile duct cell damage after liver transplantation. [104]. Another possible reason for the higher ATP levels that are induced by TMZ might be improved microcirculation at the time of reperfusion. This could increase the availability of oxygen and, therefore, facilitate ATP production. It is well known that failure of liver perfusion can impair the ability of the steatotic liver to restore ATP levels [104].

There may be drugs aimed at increasing ATP that would only be effective in steatotic livers. Compounds such as cerulenin that reduce UCP-2 expression in steatotic livers, offer protection as a result of increased availability of ATP prior to I/R [105]. However, this strategy may be ineffective in non-steatotic livers because the latter do not show an overexpression of UCP-2 [106]. Similar results have been obtained with carnitine administration [107,108]. Adiponectin down-regulated the expression of FAS and UCP2 and increased hepatic ATP levels in reduced-size liver transplantation [24].

Cyclosporin treatment during reperfusion also produced recovery of 30–40% of basal ATP, consistent with recovery of mitochondrial function [109]. Under I/R conditios, glycine and fructose given at reperfusion prevent MPT-dependent necrotic cell death. Fructose is a glycolytic substrate that protects by promoting ATP regeneration. Improving the intrahepatic content by glucose injection decreased reperfusion injury in aged liver submitted to normotermic ischemia. NO treatment suppresses MPT onset and reperfusion-induced cell killing. The NO protection is mediated by a signaling cascade of guanylyl cyclase (G-

cyclase), cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG) that blocks the MPT [110].

The activation of AMPK before ischemia using AICAR reduced ATP degradation and lactate accumulation during prolonged ischemia. This was associated with reduced biochemical and histological parameters of hepatic injury [101]. In steatotic liver transplantation, the reduction in cAMP levels induced by adenylate cyclase inhibitors (SQ22536) preserved more of the ATP and adenine nucleotide pool throughout cold ischemia period and protected steatotic livers against hepatic I/R injury [103]. This was not the case for non-steatotic liver transplantation. Indeed, in nonsteatotic liver grafts, pharmacological strategies blocking cAMP did not protect against damage [103].

CONCLUSION

Several additional points need to be addressed. The response of these protective strategies aimed at increasing ATP levels in hepatic I/R might depend on the surgical procedure. Moreover, the response of different type of liver to these treatments might differ and involve different signal transduction pathways that are at present marginally understood. Further research is required to elucidate whether the pharmacological approaches presented in this review can be translated into liver surgery associated with hepatic resections and LT. Surgical strategies such as PC have been applied in clinical surgery; however, these strategies do not exert their effects exclusively on ATP, as they affect multiple aspects of I/R injury. However, pharmacological approaches often affect ATP levels and might have systemic side effects. The hope of finding new surgical and pharmacological therapeutic applications aimed at improving post-operative outcomes during hepatic I/R processes, provides a strong impetus to identify the mechanisms responsible for the failure of ATP preservation in marginal livers. Only a full appraisal of the role of ATP in hepatic I/R and studies based in the pharmacological modulation of ATP receptors and ectonucleotidases will permit the design of new protective strategies for clinical liver surgery based on the specific regulation of energy metabolism without adverse effects. Such approaches have the potential to increase the number of organs suitable for transplantation, since they may improve the outcome for marginal grafts that would not otherwise have been transplanted, opening up new possibilities for marginal liver transplants.

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ANEXO 2

A continuación también se especifican otros artículos en los que he participado pero que no forman parte de la presente tesis:

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LIVER TRANSPLANTATION 17:1099-1110, 2011

ORIGINAL ARTICLE

Cyclic Adenosine 3′,5′-Monophosphate in Rat Steatotic Liver Transplantation

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Numerous steatotic livers are discarded as unsuitable for transplantation (TR) because of their poor tolerance of ischemia/reperfusion (I/R). Cyclic adenosine 3′,5′-monophosphate (cAMP)–elevating agents protect against I/R injury both in nonsteatotic livers that have been removed from non–heart-beating donors and subjected to warm ischemia or cold ischemia (CIS) and in perfused, isolated livers. Ischemic preconditioning (PC), which is based on brief periods of I/R, protects steatotic liver grafts, but the mechanism that is responsible is poorly understood. This study examines the role of cAMP in the vulnerability shown by steatotic livers to TR-associated I/R injury and the benefits of PC in this situation. Steatotic livers with or without PC were transplanted into Zucker rats. The hepatic levels of cAMP were measured and altered pharmacologically. Our results indicate that the cAMP levels in the nonsteatotic liver grafts were similar to those found in a sham group. However, high cAMP levels were observed in steatotic liver grafts. The blockage of cAMP generation by adenylate cyclase inhibitor pre-treatment or PC had the following results: reduced hepatic injury and increased survival of steatotic graft recipients; greater preservation of adenosine triphosphate (ATP) and reduced lactate accumulation throughout CI. This blockade of cAMP by a nitric oxide–dependent mechanism protected steatotic liver grafts against oxidative stress and microvascular disorders after reperfusion. In conclusion, cAMP blocking–based strategies could protect patients against the inherent risk of steatotic liver failure after TR. ***Liver Transpl 17:1099-1110, 2011.*** © **2011 AASLD.**

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The increasing demand for organs for transplantation (TR) has led to the acceptance of steatotic livers despite their poor tolerance of ischemia/reperfusion (I/R) injury.[1,2] The use of these marginal organs is associated with an increased risk of graft dysfunction or failure after TR.[2] In addition, many steatotic livers are discarded, and this exacerbates the critical shortage of donor livers.[1] Therefore, minimization of the adverse effects of I/R on steatotic liver TR is urgently needed.

Additional Supporting Information may be found in the online version of this article.

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine 3′,5′-monophosphate; CIS, cold ischemia; cNOS, constitutive nitric oxide synthase; DBcAMP, dibutyryl cyclic adenosine 3′,5′-monophosphate; GSH, glutathione; iNOS, inducible nitric oxide synthase; I/R, ischemia/reperfusion; Ln, lean; L-NAME, *N*(G)-nitro-L-arginine methyl ester; MDA, malondialdehyde; NAD, nicotinamide adenine dinucleotide; NO, nitric oxide; Ob, obese; $ONOO^-$, peroxynitrite; PC, ischemic preconditioning; ROS, reactive oxygen species; SOD, superoxide dismutase; SQ22536, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine; TR, transplantation; XDH, xanthine dehydrogenase; XOD, xanthine oxidase.

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2

Ischemia-Reperfusion Injury Associated with Liver Transplantation in 2011: Past and Future

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1. Introduction

Liver transplantation has evolved as the therapy of choice for patients with end-stage liver disease. However, the waiting list for liver transplantation is growing at a fast pace, whereas the number of available organs is not growing at a proportional rate. The potential use of steatotic livers for transplant, one of the most common types of organs from marginal donors, has become a major focus of investigations. However the clinical problem is still unresolved since steatotic livers are more susceptible to ischemia-reperfusion (I/R) injury and, when used, have poorer outcome than non-steatotic livers. Indeed, the use of steatotic livers for transplantation is associated with increased risk of primary non-function or dysfunction after surgery. Therefore, minimizing the adverse effects of I/R injury could improve outcomes in steatotic liver surgery, increase the number both of suitable transplantation grafts and of patients who successfully recover from liver transplantation.

The present review focuses on the complexity of hepatic I/R injury, summarizing conflicting results obtained from the literature about the mechanisms responsible for it. We also review the therapeutic strategies designed in past years to reduce I/R injury, attempting to explain why most of them have not been applied clinically. Finally, we will consider new potential protective strategies that have shown promising results for I/R injury with the potential to increase the number of liver suitable for liver transplantation.

2. Hepatic ischemia-reperfusion injury associated with liver transplantation. An unresolved problem in clinical practice

Liver transplantation (LT) dates back to 1963, when Thomas Starzl carried out the first transplant on a child suffering from biliary atresia. LT has evolved as the therapy of choice for patients with end-stage liver disease. However, I/R injury, inherent in every LT, is the main cause of both initial poor function and primary non-function of liver allograft. The latter is responsible for 81% of re-transplantations during the first week after surgery (Clavien et al., 1992; Jaeschke, 1996). I/R injury is a phenomenon whereby cellular damage in a hypoxic organ is accentuated following the restoration of oxygen delivery (Jaeschke, 1998; Teoh et al., 2003; Jaeschke, 2003). In the liver, this form of injury was recognized as a

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Review Article

The Current State of Knowledge of Hepatic Ischemia-Reperfusion Injury Based on Its Study in Experimental Models

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The present review focuses on the numerous experimental models used to study the complexity of hepatic ischemia/reperfusion (I/R) injury. Although experimental models of hepatic I/R injury represent a compromise between the clinical reality and experimental simplification, the clinical transfer of experimental results is problematic because of anatomical and physiological differences and the inevitable simplification of experimental work. In this review, the strengths and limitations of the various models of hepatic I/R are discussed. Several strategies to protect the liver from I/R injury have been developed in animal models and, some of these, might find their way into clinical practice. We also attempt to highlight the fact that the mechanisms responsible for hepatic I/R injury depend on the experimental model used, and therefore the therapeutic strategies also differ according to the model used. Thus, the choice of model must therefore be adapted to the clinical question being answered.

1. Introduction

Ischemia-reperfusion (I/R) injury is a phenomenon in which cellular damage in a hypoxic organ is accentuated following the restoration of oxygen delivery [1–3]. In the liver, this form of injury was recognized as a clinically important pathological disorder by Toledo-Pereyra et al. in 1975 during studies of experimental liver transplantation (LT). However, it was not until the mid-1980s that the term reperfusion injury was generally used in the literature on LT [2]. I/R injury is an important cause of liver damage occurring during surgical procedures including hepatic resections and LT [4–6]. The shortage of organs has led centers to expand their criteria for the acceptance of marginal grafts that exhibit poor tolerance to I/R [7]. Some of these include the use of organs from older donors and grafts such as small-for-size or steatotic livers. However, I/R injury is the underlying cause of graft dysfunction in marginal organs [7]. Indeed, the use of steatotic livers for transplantation is associated with an increased risk of primary nonfunction or dysfunction after surgery [8]. In addition, the occurrence of postoperative liver failure after hepatic resection in a steatotic liver exposed to normothermic ischemia has been reported [9]. Therefore, minimizing the adverse effects of I/R injury could improve outcomes in steatotic liver surgery, increasing the number of patients who successfully recover from major liver surgery.

Animal models of cold and warm hepatic I/R are valuable tools for understanding the physiopathology of hepatic I/R injury and discovering novel therapeutic targets and drugs. Some of the mechanisms and cell types involved in hepatic I/R injury are described below.

The lack of oxygen in hepatocytes during ischemia causes ATP depletion and alterations in H^+, Na^+, and Ca^{2+} homeostasis that activate hydrolytic enzymes and impair cell volume regulation leading to the swelling of sinusoidal endothelial cells (SECs) and Kupffer cells (KCs) [10]. This fact, together with the imbalance between nitric oxide (NO) and endothelin production, contributes to the narrowing of the

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AICAR and Trimetazidine in University of Wisconsin Solution are Effective to Increase Survival in Recipients Transplanted with Steatotic Livers

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Abstract

Background & Aims: The development of protective strategies for steatotic liver grafts is vital in order to increase the number of organs available for transplantation. We evaluated the usefulness of trimetazidine (TMZ) and aminoimidazole-4-carboxamide ribonucleoside (AICAR) (separately or in combination) as new additives to University of Wisconsin (UW) solution in steatotic liver transplantation. We also investigated the underlying mechanisms responsible for this protection.

Methods: Steatotic livers preserved in UW solution with TMZ and AICAR (separately or in combination) were transplanted into Zucker rats. Adenosine monophosphate activated protein kinase (AMPK) and nitric oxide (NO) were inhibited pharmacologically (n=10 transplantations, n=20 rats for each intervention).

Results: The addition of TMZ and AICAR (separately or in combination) to UW solution increased AMPK and nitrates and nitrites, up-regulated ERK 1/2, reduced oxidative stress, transaminases and damage score and improved survival in recipients. The following values were obtained: ERK 1/2 expression (111.9±4.06 and 219.9±17.90, p=0.000), malondialdehyde levels (1.65±0.03 and 0.23±0.01, p=0.000) and survival in recipients (30% and 60%, p=0.01) for steatotic livers preserved in UW and UW+TMZ+AICAR, respectively. The benefits of TMZ and AICAR were abolished when AMPK or NO were inhibited.

Conclusion: TMZ and AICAR may be useful new additives to UW solution in steatotic liver transplantation whereas a combination of the two was unnecessary. TMZ activated AMPK and this in turn increased NO, reduced oxidative stress and up-regulated ERK 1/2, thus protecting steatotic liver grafts and improving post-transplant outcomes.

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Key words

liver transplantation; steatotic liver; ischemia-reperfusion; preservation solutions

Introduction

Up to 30% of all livers retrieved for organ transplantation exhibit steatotic transformations.[1,2] The increasing demand for organs for transplantation has led to the acceptance of steatotic livers, which have poor tolerance to ischemia-reperfusion (I/R) injury.[3,4] The use of these marginal organs for transplantation is associated with an increased risk of graft dysfunction or failure after surgery.[4] In addition, many steatotic livers are discarded for transplantation, exacerbating the critical shortage of donor livers.[3] As a result, there is an urgent need to minimize the adverse effects of I/R in steatotic liver transplantation.

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Trimetazidine (TMZ), introduced as an anti-ischemic drug in the heart,[5,6] has been used by our group as a new additive in UW solution to protect steatotic livers exposed to prolonged cold ischemia (24 h) in an *ex-vivo* model of hepatic ischemia.[7,8]

Nitric oxide (NO) plays an important protective role in a variety of organs undergoing I/R injury.[9-11] Previous results have indicated that reduction of oxidative stress and overexpression of mitogen-activated protein kinase ERK 1/2 may be involved in the benefits of NO. The beneficial effects of NO on oxidative stress in livers undergoing warm ischemia have previously been reported.[12,13] In addition, in hearts subjected to I/R injury, the protection conferred by several strategies has been associated with ERK 1/2 activation in a NO-dependent manner.[14,15] Given these observations and the previous results indicating the involvement of NO in the benefits of TMZ in experimental models of I/R,[8,16] the possibility that the benefits of TMZ in steatotic liver transplantation could be mediated by NO should not be discarded.

A relationship between adenosine monophosphate activated protein kinase (AMPK) and NO was previously described.[17-19]

Chapter 6

Experimental Models in Liver Surgery

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Additional information is available at the end of the chapter

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1. Introduction

Ischemia-Reperfusion (I/R) injury is an important cause of liver damage occurring during surgical procedures including hepatic resections and liver transplantation (LT) [1-3]. The shortage of organs has led centers to expand their criteria for the acceptance of marginal grafts that exhibit poor tolerance to I/R [4]. Some of these include the use of organs from older donors and grafts such as small-for-size or steatotic livers. However, I/R injury is the underlying cause of graft dysfunction in marginal organs [4]. Indeed, the use of steatotic livers for transplantation is associated with an increased risk of primary nonfunction or dysfunction after surgery [5]. In addition, the occurrence of postoperative liver failure after hepatic resection in a steatotic liver exposed to normothermic ischemia has been reported [6]. A large number of factors and mediators play a part in liver I/R injury. The relationships between the signalling pathways involved are highly complex and it is not yet possible to describe, with absolute certainty, the events that occur between the beginning of reperfusion and the final outcome of either poor function or a non-functional liver graft. We will show that the mechanisms responsible for hepatic I/R injury depends on the experimental model used, who are valuable tool for understanding the physiopathology of hepatic I/R injury and discovering novel therapeutic targets and drugs. Several strategies to protect the liver from I/R injury have been developed in animal models and, some of these, might find their way into clinical practice. The species used for experimental investigation of hepatic I/R injury range from mice to pigs. The book chapter will discuss the numerous experimental models used to study the complexity of hepatic I/R injury, data reported in choice of the animal model, when selecting an animal species, the age, the sex, the degree of steatosis…etc. Thus, the different strengths and limitations of the different experimental models will be discussed. Also the standardized experimental conditions, such as anesthetic and analgesic procedures will be described. We also attempt to highlight the fact that the types of ischemia (cold and warm ischemia) play an important role in experimental liver surgery. The most

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Tauroursodeoxycholic Acid Affects PPARγ and TLR4 in Steatotic Liver Transplantation

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Numerous steatotic livers are discarded for transplantation because of their poor tolerance to ischemia-reperfusion (I/R). We examined whether tauroursodeoxycholic acid (TUDCA), a known inhibitor of endoplasmic reticulum (ER) stress, protects steatotic and nonsteatotic liver grafts preserved during 6 h in University of Wisconsin (UW) solution and transplanted. The protective mechanisms of TUDCA were also examined. Neither unfolded protein response (UPR) induction nor ER stress was evidenced in steatotic and nonsteatotic liver grafts after 6 h in UW preservation solution. TUDCA only protected steatotic livers grafts and did so through a mechanism independent of ER stress. It reduced proliferator-activated receptor-γ (PPARγ) and damage. When PPARγ was activated, TUDCA did not reduce damage. TUDCA, which inhibited PPARγ, and the PPARγ antagonist treatment up-regulated toll-like receptor 4 (TLR4), specifically the TIR domain-containing adaptor inducing IFNβ (TRIF) pathway. TLR4 agonist treatment reduced damage in steatotic liver grafts. When TLR4 action was inhibited, PPARγ antagonists did not protect steatotic liver grafts. In conclusion, TUDCA reduced PPARγ and this in turn up-regulated the TLR4 pathway, thus protecting steatotic liver grafts. TLR4 activating-based strategies could reduce the inherent risk of steatotic liver failure after transplantation.

Key words: ER tress, PPAR-γ, steatotic liver grafts, TLR4, transplantation, TUDCA

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; CHOP, C/EBP homologous protein; eIF2α, eukaryotic translation initiation factor 2 subunit α; ER, endoplasmic reticulum; GRP78, 78-kDa glucose-regulated/binding immunoglobulin protein; GW9662, 2-chloro-5-nitro-*N*-phenylbenzamide; HTK, histidine tryptophan ketoglutarate; IRE1, inositol-requiring enzyme 1; I/R, ischemia-reperfusion; Ln, lean; MDA, malondialdehyde; MPL-A, monophosphoryl lipid A; MyD88, myeloid differentiation factor 88; Ob, obese; PPAR-γ, peroxisome proliferator activated receptor-γ; Rosiglitazone, (*RS*)-5-[4-(2-[methyl(pyridine-2-yl)-amino]ethoxy)benzyl]thiazolidine-2,4-dione; ROS, reactive oxygen species; TLR4, toll-like receptor 4; TR, transplantation; TRAF2, tumor necrosis factor-associated factor 2; TRIF, TIR domain-containing-adaptor inducing IFN-β; TUDCA, tauroursodeoxycholic acid; UPR, unfolded protein response; UW, University of Wisconsin; XBP-1, X-box-binding protein 1.

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Introduction

Ischemia-reperfusion (I/R) injury is a major cause of early graft dysfunction after liver transplantation. Steatosis is currently estimated to be present in up to 50% of deceased donor livers and is recognized as the key donor variable predicting posttransplant outcomes (1–3). The use of steatotic livers for transplantation is associated with increased risk of graft dysfunction or failure after surgery. In addition, many steatotic livers are discarded for transplantation, exacerbating the critical shortage of donor livers (1,4,5).

Endoplasmic reticulum (ER) stress is emerging as an important component of inflammatory responses in the liver associated with I/R processes (6,7). In response to ER stress, a signal transduction cascade termed "the unfolded protein response (UPR)" is induced (8,9). The UPR has three branches: inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK) and activating transcription factor (ATF6). These proteins are normally held in inactive states in ER membranes by binding to intra-ER chaperones, particularly the 78-kDa glucose-regulated/binding immunoglobulin protein (GRP78). When injury is excessive, these ER stress

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Chapter 4

ADENOSINE TRIPHOSPHATE IN EXPERIMENTAL LIVER SURGERY

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ABSTRACT

The shortage of organs has led centers to the acceptance of marginal grafts such as fatty livers, small-for-size liver or aged donors. However, the clinical problem is unresolved since this type of liver tolerates poorly hepatic ischemia-reperfusion (I/R) and show regenerative failure after liver surgery. The use of marginal liver for transplant is associated with increased risk of primarily non-function or dysfunction after surgery, being the deficiencies in energy metabolism one of the main mechanisms responsible for the vulnerability of this liver type to I/R injury and regenerative failure. Indeed, experimental studies and clinical observations clearly indicate that marginal livers show more adenosine triphosphate (ATP) depletion during ischemia and synthesize less ATP than normal livers during the early phase of reperfusion. This book chapter will be focused on the role of ATP in hepatic I/R injury and the mechanisms responsible of ATP depletion in both marginal and normal livers. We will show that the deleterious effects of ischemia on ATP depletion and the lactate production limit survival of hepatocytes, being this effect more exacerbated in marginal livers. Also, we will explain how different conditions, including the presence of fatty infiltration or starvation, affect ATP recovery during reperfusion, a prerequisite for liver graft viability after surgery. In hepatic I/R injury cell death can occur via necrosis or apoptosis. We will review the key role of ATP as a putative apoptosis/necrosis switch: when ATP depletion is severe, necrosis ensues before the activation of the energy-requiring apoptotic pathway. The present book chapter will discuss how ATP depletion and its posterior restoration depends on the type of ischemia (cold or warm ischemia), the type of liver, duration and the extent of hepatic ischemia, starvation, and the presence of liver regeneration. We will show that the mechanism responsible for ATP recovery during reperfusion depends of the experimental model used. Therefore, is very important to choose to standardize experimental conditions according to the clinical question being

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Chapter 4

Expanding the Donor Pool in Liver Transplantation: Influence of Ischemia-Reperfusion

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Abstract

Improvements in surgical techniques, immunosuppression, and patient management have led to the optimization of liver transplantation outcomes. However, the waiting list for liver transplantation is increasing at a greater pace. The large imbalance between the growing pool of potential liver transplant recipients and the scarcity of donor organs has fueled efforts to maximize existing donors and identify new sources.

To expand the potential donor pool, clinical, and organ procurement agencies are continually modifying the criteria of an acceptable liver donor and are looking to marginal or expanded donors to meet the waiting list demands. This book chapter will be focused on the current state of liver transplantation using grafts from extended criteria donors (elderly donors, steatotic donors, donors with malignancies, donors with viral hepatitis) and from donation after cardiac death (non-heart beating donors), as well as the use of partial grafts (split grafts and living-donor liver transplantation) and other suboptimal donors (donors with hypernatraemia, infections, hypotension and inotropic support). Overall, broadened criteria for acceptable donor livers appear to lessen graft survival rates somewhat compared with rates for ideal donor organs.

Donors are generally considered marginal if there is a risk of initial poor function or primary non-function. The present book chapter will discuss the factors defining marginality of a graft, the pathophysiology of the marginal donor, and the issues faced by transplant units in making the decision to use such a graft; along with strategies for minimizing the ischemia-reperfusion injury experienced by the organs. We will show the

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Chapter 2

NEUROLOGICAL COMPLICATIONS IN LIVER TRANSPLANTATION

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ABSTRACT

Over the past two decades, remarkable advances have been made in the field of liver transplantation and improvements in surgical techniques and perioperative care have reduced the mortality and morbidity associated with liver transplantation. However the neurological complications associated with liver transplantation are one of the main unresolved problems in clinical practice.

We will present the relationship between the functional status of the liver and that of the brain. This book chapter will be focus on hepatic encephalopathy, the major neurologic complications manifestation, followed by seizures and immunosuppression toxicity. The differential neurological complications caused by the different toxins or immunosuppressors as well as the neurotoxic mechanisms will be also described in the present book chapter. We will also discuss why the reported incidence of neurological complications is variable for different transplant centers.

We will show that neurologic complications of liver transplantation are more common than other solid organ transplant with a significantly lower incidence in living donor liver transplantation versus patients who receive a liver graft from cadaveric donor. We will also show that the occurrence of neurological complications in pediatric transplantation is significantly lower than in adult transplantation.

The knowledge of neurological complications of liver transplantation is important for transplantation teams to reduce their prevalence and improve their management. At this time their management is empirical, being currently based more on clinical practical experience than on evidence derived from scientific literature or animal models. Thus, more studies should be development for prevention and treatment of neurological complications.

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Research Article

EASL JOURNAL OF HEPATOLOGY

Adiponectin and resistin protect steatotic livers undergoing transplantation

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Background & Aims: Numerous steatotic livers are discarded for transplantation because of their poor tolerance to ischemia-reperfusion. Controversial roles for adiponectin and related adipocytokines visfatin and resistin have been described in different liver pathologies, nevertheless it is unknown their possible implication in ischemia-reperfusion injury associated with liver transplantation. Our study aimed at characterizing the role of the adiponectin-derived molecular pathway in transplantation with steatotic and non-steatotic liver grafts.

Methods: Steatotic and non-steatotic liver transplantation was carried out and the hepatic levels of adiponectin, visfatin and resistin were measured and modulated either pharmacologically or surgically.

Results: Steatotic liver grafts exhibited downregulation of both adiponectin and resistin when subjected to transplantation. Adiponectin pre-treatment only protected steatotic grafts and did it so through a visfatin-independent and resistin-dependent mechanism. Adiponectin-derived resistin accumulation activated the PI3K/Akt pathway, unravelling AMPK as an upstream mediator of adiponectin's actions in steatotic grafts. Strategies aimed at increasing adiponectin including either AMPK activators or the induction of ischemic preconditioning (which activates AMPK) increased resistin accumulation, prevented the downregulation of PI3K/Akt pathway and protected steatotic liver grafts. Conversely, PI3K/Akt pathway upregulation and hepatic protection induced by adiponectin were abolished when resistin action was inhibited.

Conclusions: Our findings reveal a new protective pathway in steatotic liver transplantation, namely AMPK-adiponectin-resistin-PI3K/Akt, which may help develop new strategies aimed at increasing either adiponectin or resistin in the steatotic liver undergoing transplant to ultimately increase organ donor pool and reduce waiting list.

Keywords: Adiponectin; Resistin; Visfatin; Steatotic liver grafts; Liver transplantation.

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Abbreviations: I/R, ischemia-reperfusion; PC, ischemic preconditioning; Ob, obese; Ln, lean; TR, transplantation; UW, University of Wisconsin; AMPK, AMP-activated protein kinase; AICAR, aminoimidazole-4-carboxamide ribonucleoside; PI3K, phosphoinositide-3-kinase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; α-GST, alpha-glutathione S-transferase; H&E, hematoxylin and eosin; ANOVA, analysis of variance.

Introduction

Owing to the discrepancy between organ donation and the demand for liver transplantation, expanding the liver donor pool is of vital importance. The potential use of steatotic livers, which are among the most common types of organs from extended-criteria donors, has become a major focus of investigation for transplantation. Unfortunately, more than 50% of livers that are considered as not suitable for transplantation due to their pathological conditions are discarded because of the presence of fatty infiltration. Steatotic livers are more susceptible to ischemia/reperfusion (I/R) injury, and when they are transplanted, they lead to poorer outcomes in comparison with non-steatotic livers. Indeed, the use of steatotic livers for transplantation is associated with an increased risk of primary non-function or dysfunction after surgery [1,2].

It has been suggested that the pathogenesis of fatty liver diseases is associated with deregulated production and release of novel adipocytokines, including adiponectin, visfatin and resistin [3]. Since the discovery of adiponectin, diverse experimental and clinical studies have demonstrated that adiponectin mediates anti-inflammatory effects [4–6]. To the best of our knowledge, only 2 studies have reported a role for adiponectin in steatotic livers subjected to I/R. Massip-Salcedo et al. [7] showed injurious effects of adiponectin on steatotic livers subjected to 60 min of warm ischemia. However, Man et al. [4] demonstrated the anti-inflammatory effects of adiponectin in small fatty grafts subjected to 40 min of cold ischemia. The aforementioned data suggest that the role of adiponectin depends on the surgical conditions. Therefore, one of the aims of the present study was to determine whether adiponectin should be inhibited or activated

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Research Article

JOURNAL OF HEPATOLOGY

Resistin and visfatin in steatotic and non-steatotic livers in the setting of partial hepatectomy under ischemia-reperfusion

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Background & Aims: This study examined whether the regulation of resistin and visfatin could reduce damage and improve regeneration in both steatotic and non-steatotic livers undergoing partial hepatectomy under ischemia-reperfusion, a procedure commonly applied in clinical practice to reduce bleeding.

Methods: Resistin and visfatin were pharmacologically modulated in lean and obese animals undergoing partial hepatectomy under ischemia-reperfusion.

Results: No evident role for these adipocytokines was observed in non-steatotic livers. However, obese animals undergoing liver surgery showed increased resistin in liver and plasma, without changes in adipose tissue, together with visfatin downregulation in liver and increment in plasma and adipose tissue. Endogenous resistin maintains low levels of visfatin in the liver by blocking its hepatic uptake from the circulation, thus regulating the visfatin detrimental effects on hepatic damage and regenerative failure. Indeed, the administration of anti-resistin antibodies increased hepatic accumulation of adipocyte-derived visfatin, exacerbating damage and regenerative failure. Interestingly, treatment with anti-visfatin antibodies protected steatotic livers, and similar results were obtained with the concomitant inhibition of resistin and visfatin. Thus, when visfatin was inhibited, the injurious effects of anti-resistin antibodies disappeared. Herein we show that upregulation of visfatin increased NAD levels in the remnant steatotic liver, whereas visfatin inhibition decreased them. These later observations suggest that visfatin may favour synthesis of NAD instead of DNA and induces alterations in amino acid metabolism-urea cycle and NO production, overall negatively affecting liver viability.

Conclusions: Our results indicate the clinical potential of visfatin blocking-based therapies in steatotic livers undergoing partial hepatectomy with ischemia-reperfusion.

Keywords: Liver surgery; Hepatic steatosis; Regeneration; Resection; Adipocytokines; Ischemia-reperfusion.

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Abbreviations: PH, partial hepatectomy; I/R, ischemia-reperfusion; Nampt, nicotinamide phosphoribosyltransferase; NAD, nicotinamide adenine dinucleotide; TNF, tumor necrosis factor; IL, interleukin; Ob, obese; Ln, lean; BrdU, bromo-deoxyuridine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HGF, hepatocyte growth factor; TGF, transforming growth factor; MPO, myeloperoxidase; MDA, malondialdehyde; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; GDH, glutamate dehydrogenase; CPT2, carnitine palmytoil transferase-2; MCAD, medium chain acyl-CoA dehydrogenase; FGF21, fibroblast growth factor-21; SOD2, superoxide dismutase-2; GPX1, glutathione peroxidase-1; SCD1, stearoyl-CoA desaturase-1; FAS, fatty acid synthase; COX1, cytochrome *c* oxidase subunit I; COXIV, cytochrome *c* oxidase subunit IV.

Introduction

In clinical situations, partial hepatectomy (PH) under ischemia-reperfusion (I/R) is usually performed to control bleeding during parenchymal dissection [1]. More than 20% of patients coming to liver resection have some degree of steatosis, usually related to obesity [2,3]. Hepatic steatosis is a major risk factor for liver surgery since it is associated with an increased complication index and postoperative mortality after major liver resection [4].

Under pathological conditions, adipose tissue has appeared as a highly active endocrine gland, secreting adipocytokines such as resistin and visfatin [5]. However, resistin and visfatin are also expressed in liver under obesity conditions and during fibrosis [6,7].

Increased levels of resistin have been reported in patients with cirrhosis, correlating with the severity of the disease [5,8]. Opposite effects of resistin on myocardial injury have been reported [9].

Visfatin, also known as nicotinamide phosphoribosyltransferase (Nampt), is critical for the synthesis of nicotinamide adenine

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THE EFFECTS OF GLUCOSE AND LIPIDS IN STEATOTIC AND NON-STEATOTIC LIVERS IN CONDITIONS OF PARTIAL HEPATECTOMY UNDER ISCHEMIA-REPERFUSION

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Running Title: Glucose and lipids in partial hepatectomy under I/R

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